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(57) Abstract

The invention discloses lipophilic oligonucleotide analogs that are capable of efficient passive diffusion across cell membranes. These oligonucleotides contain at least two nucleotide residues and have an octanol:water partition coefficient of about -0.3 to + 2.5 and a solubility in water of at least 0.001 μg/mL. Invention embodiments which include lipophilic oligonucleotide analogs having either at least 60 % of the internucleotide linkages are lipophilic, or at least 60 % of the bases contain lipophilic substitutions, or at least 60 % of the sugars contain lipophilic substitutions, or a combination of these sums to 60 %. These oligonucleotides may be conjugated to a label and used to visualize cells or subcellular compartments.

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LIPOPHILIC OLIGONUCLEOTIDE ANALOGS

Field of the Invention

The invention is directed to oligonucleotide analogs, in particular to lipophilic oligonucleotide analogs that efficiently enter cell cytoplasm or cross membranes without the aid of either transfection compounds or other agents or techniques.

Background of the Invention

The invention relates to oligonucleotide analogs which are capable of passive permeation of cell membranes and synthetic intermediates therefor as well as their use in cell staining, diagnostic and therapeutic applications.

Workers have described oligonucleotide analogs, complexes of oligonucleotide analogs and nucleoside analogs having lipophilic or related modifications for enhancing their delivery into cells, increasing nuclease stability or other purposes (see e.g., WO 96/15778; WO 96/07392; WO 96/05298; WO 96/04788; WO 90/10448; WO 89/12060; EP 462 145 B1; EP 092 574 B1; U.S. Patent Nos. 5,420,330, 4,958,013, 4,904,582; Agrawal et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 7079-7083 1988; Dagle et al., *Nucl. Acids Res.* 19:1805-1810 1991; Matteucci et al., *Nucl. Acids Res.* 16:4831-4839 1988; Huang et al., *J. Org. Chem.* 56:3869-3882 1991; Huff et al., *J. Biol. Chem.* 262:12843-12850 1987; Inoue et al., *Nucl. Acids Res.* 15:6131-6148 1987; Itoh et al., *Heterocycles* 17:305-309 1982; Itoh et al., *Nucleosides & Nucleotides* 1:179-190 1982; Kabanov et al., *FEBS* 259:327-330 1990; Manoharan et al., *Tet. Lett.* 32:7171-7174 1991; Montgomery et al., *J. Het. Chem.* 14:195-197 1977; Montgomery et al., *J. Med. Chem.* 10:165-167 1967; Mizuno et al., *J. Org. Chem.* 28:3329-3331 1963; Praseuth et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 85:1349-1353 1988; Schneider et al., *Tet. Lett.* 31:335-338 1990; Severin et al., *Adv. Enz. Regul.* 31:417-430 1991; Shoji et al., *Nucl. Acids Res.* 19:5543-5550 1991; Singer et al., *Biochem.* 28:1478-1483 1989; Sproat et al., *Nucl.*

Acids Res. 17:3373-3386 1989; Tanaka et al., *Tet. Lett.* 19:4755-4758 1979; Uhlmann et al., *Chem. Revs.* 90:543-585 1990; Wright et al., *J. Med. Chem.* 30:109-116 1987.

Workers have described permeation of molecules, including oligonucleotides, across lipid membranes or into cells (see e.g., Hansch et al., *J. Pharm. Sci.* 61:1-19 1972; Lieb et al., *Nature* 224:240-243 1969; Loke et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 86:3474-3478 1989; W.D. Stein *New Comprehensive Biochemistry*, vol 2, *Chapter 1: Permeability for Lipophilic Molecules, Membrane Transport* Elsevier/North-Holland Biomedical Press, Amsterdam, S.L. Bonting et al. editors, 1981, pages 1-28; Walter et al., *J. Membrane Biol.* 90:207-217 1986; Yakubov et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 86:6454-6458 1989).

Objects of the Invention

The invention compositions or methods include one or more compounds or methods that accomplish one or more of the following objects.

It is an object of the invention to provide lipophilic oligonucleotide analogs and intermediates for making them.

Another object of the invention is to provide lipophilic oligonucleotide analogs that are suitable for permeation into cell cytoplasm or cell nuclei *in vitro* or *in vivo* in the presence or absence of serum or blood.

Another object is to provide lipophilic oligonucleotide analogs that are suitable for staining one or more subcellular organelles or compartments.

Another object of the invention is to provide lipophilic oligonucleotide analogs that are suitable for tagging or marking items or compounds.

Another object is to provide methods to deliver lipophilic oligonucleotide analogs into cells *in vitro* or *in vivo*.

Another object is to provide compositions comprising lipophilic oligonucleotide analogs bonded to a detectable label.

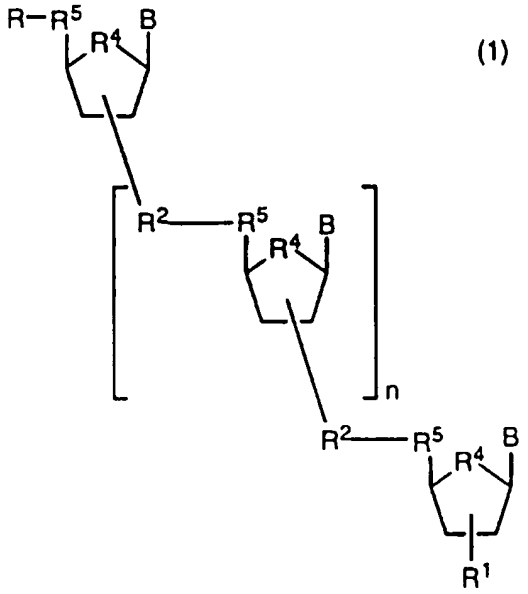
Summary of the Invention

The invention is directed to oligonucleotides capable of passive diffusion across mammalian cell or organelle membranes or any other cell membrane (plant, parasite, bacterial, yeast, viral, or fungal).

Invention oligonucleotide analogs are characterized by oligonucleotides comprising internucleotide linkages, bases and sugars wherein the oligonucleotide has a Log value of the octanol:water partition coefficient of

-0.3 to +2.5 and a solubility in water of at least 0.001 μg/mL, and the and salts, solvates and hydrates thereof.

Invention embodiments include oligonucleotide analogs having structure (1)



(1)

wherein

R is OH, blocked OH, $N(R^{14})_2$, $P(O)(R^{15})_2$, or a linker;

R^1 is an oligonucleotide, a blocking group, OH, $N(R^{14})_2$, $P(O)(R^{15})_2$, a solid support, or a linker bonded to the 2' or 3' position of a furanose ring (or its carbocyclic analog), and the remaining 2' or 3' position is substituted with R^3;

each R^2 independently is an internucleotide linkage bonded to the 2' or 3' position, and the remaining 2' or 3' position is substituted with R^3;

each R^3 independently is H, OH, F, blocked hydroxyl, $N(R^{14})_2$, -O-alkyl (C_{1-8}), -O-alkyl (C_{1-8}) where the alkyl group is substituted with halogen, hydroxyl or oxygen, -O-alkenyl (C_{3-8}), -S-alkyl (C_{1-8}) or a linker;

each R^4 independently is O or CH_2;

each R^5 independently is CH_2, NR^6, O, S, SO, SO_2;

each R^6 independently is H, alkyl (C_{1-6}) or alkyl (C_{1-6}) where the alkyl group is substituted with halogen, hydroxyl or oxygen;

each R^{14} independently is hydrogen, a protecting group, hydrocarbyl, or pseudohydrocarbyl;

each R^{15} independently is hydroxyl (OH), blocked hydroxyl, SH, blocked SH, or $N(R^{14})_2$;

n is an integer from 0 to 48; and

each B independently is a base, wherein the total number of bonded monomers designated by the variable n plus any oligonucleotide at R^1 is 2-50.

The invention oligonucleotide analogs are useful to visualize or stain cells and are thus useful in a method comprising: contacting cells to be visualized with the oligonucleotide under conditions wherein diffusion across the cell membrane can occur so as to internalize said oligonucleotide within the cells; removing from the cells any oligonucleotide which has not diffused across the membrane and become internalized; and detecting the oligonucleotide which has been internalized in the cells to visualize the cells.

The invention oligonucleotide analogs are useful as agents to deliver oligonucleotides into cells and are thus useful in a method comprising: contacting a cell with an invention oligonucleotide.

Brief Description of the Drawings

Figure 1 shows a standard curve for the determination of partition coefficient based on retention time in RPLC.

Figure 2 shows the chemical structures of oligonucleotide analogs used to visualize cells.

Detailed Description of the Invention

Definitions. Halogen means F (fluorine), Cl (chlorine), Br (bromine) or I (iodine).

Alkyl means linear, branched or cyclic saturated hydrocarbons.

Alkenyl means linear, branched or cyclic unsaturated hydrocarbons where one or more double bonds are present.

Alkynyl means linear, branched or cyclic unsaturated hydrocarbons where one or more triple bonds are present.

As used herein, hydrocarbyl groups contain only carbon and hydrogen and includes alkyl, alkenyl or alkynyl groups. Hydrocarbyl groups typically contain 1, 2 ,3 ,4, 5, 6, 7 or 8 carbon atoms, but includes groups having more than 8 carbon atoms, such as groups containing 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 carbon atoms.

As used herein, psuedohydrocarbyl groups are hydrocarbyl substituents which contain one or more heteroatoms (including those present as substituents) representing less than 50% of the total non-hydrogen atoms in the pseudohydrocarbyl substituent. Typically, pseudohydrocarbyl groups

bonded to invention oligonucleotide analogs, including structure (1) oligonucleotides, contain 1-18 carbon atoms, usually 3-12. Pseudohydrocarbyl moieties optionally contain 1, 2, 3 or 4 heteroatoms. Heteroatoms usually found in pseudohydrocarbyl groups are O, N, S or halogen. The heteroatoms may be present as an ether, ketone, hydroxyl, thiol (SH), protected thiol, primary amine, secondary amine, tertiary amine, protected primary amine, amide, thioether (-S-), carboxyl, protected carboxyl, nitro (NO_2), azido (N_3), ester (-C(O)-OR^X where R^X is hydrocarbyl or pseudohydrocarbyl), carbonate (-O-C(O)-OR^X) or carbamate (-O-C(O)-$NR^{14}R^X$). When an amine or carboxyl is present, the group will potentially carry a partial or full charge at physiological pH, and sufficient carbon atoms, generally about 12-18, will generally need to be present in the pseudohydrocarbyl group to offset the hydrophilic character of the charge. Generally, pseudohydrocarbyl substituents that facilitate passive diffusion decrease the polarity or increase the lipophilicity of the parent oligonucleotide and do not carry any charged atoms or groups, unless more than about 14 carbon atoms are present.

As used herein "base" means protected and unprotected purine, pyrimidine heterocycles found in nucleic acids or their modified forms. Modifications include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles previously described (see, e.g., PCT US94/10539). Bases suitable for use herein include alkylated purines or pyrimidines, acylated purines or pyrimidines, or other analogs of purine or pyrimidine bases and their aza and deaza analogs. Exemplary bases include N^4,N^4-ethanocytosine, 7-deazaxanthosine, 7-deazaguanosine, 8-oxo-N^6-methyladenine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, inosine, N^6-isopentenyl-adenine, 1-methyladenine, 2-methylguanine, 5-methylcytosine, N^6-methyladenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy aminomethyl-2-thiouracil, 5-methoxyuracil, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-(1-propynyl)-4-thiouracil, 5-(1-propynyl)-2-thiouracil, 5-(1-propynyl)-2-thiocytosine, 5-(1-butynyl)-4-thiouracil, 5-(1-butynyl)-2-thiouracil, 5-(1-butynyl)-2-thiocytosine, 2-thiothymidine, and 2,6-diaminopurine. In addition to these base analogs, one can incorporate pyrimidine analogs including 6-azacytosine, 6-azathymidine and 5-trifluoromethyluracil described in WO 92/02258 into the invention oligonucleotides. Typically bases are adenine, guanine, thymine, uracil, cytosine, 5-methylcytosine, 5-(1-propynyl)uracil, 5-(1-propynyl)cytosine, 8-oxo-N^6-methyladenine, 7-deaza-7-methylguanine, 7-

deaza-7-methyladenine, 7-deazaxanthosine, 7-deaza-7-(1-propynyl)adenine, 7-deaza-7-(1-propynyl)guanine, 7-deaza-7-(1-butynyl)adenine, 7-deaza-7-(1-butynyl)guanine, 5-(1-butynyl)uracil, 5-(1-butynyl)cytosine.

"Nucleoside," "nucleotide" and "monomer" include those moieties which contain both the common purine and pyrimidine bases adenine, guanine, cytosine, thymine and uracil, and modified bases or analogs thereof, particularly either lipophilic analogs or analogs that enhance binding affinity for complementary nucleic acid sequences. Monomers, nucleosides or nucleotides are bonded together to form the invention oligonucleotide analogs. The terms "nucleoside," "nucleotide" and "monomer" are generic to ribonucleosides or ribonucleotides, deoxyribonucleosides or deoxyribonucleotides, or to any other nucleoside which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Thus, the stereochemistry of the sugar carbons may be other than that of D-ribose in one or more residues. Also included are oligonucleotide-like compounds or analogs where the ribose or deoxyribose moiety is replaced by an alternate structure such as the 6-membered morpholino ring described in U.S. patent number 5,034,506 or where an acyclic structure serves as a scaffold that positions the base or base analogs in a manner that permits efficient binding to target nucleic acid sequences or other targets. Oligonucleotide-like compounds with acyclic structures in place of the sugar residue and/or the linkage moiety are specifically intended to include both (i) structures that serve as a scaffold that positions bases or base analogs in a manner that permits efficient sequence-specific binding to target nucleic acid base sequences and (ii) structures that do not permit efficient binding or hybridization with complementary base sequences. Elements ordinarily found in oligonucleotides, such as the furanose ring or the phosphodiester linkage may be replaced with any suitable functionally equivalent element.

Linkage and internucleotide linkage mean an uninterrupted chain of atoms that bond adjacent monomers or nucleotides together. Linkage includes unmodified phosphodiester linkages, -O-P(O)(OH)-O-, and modified or substitute linkages. Substitute linkage means a linkage other than a phosphodiester linkage that links the sugar or sugar analog of adjacent monomers or nucleotides. Substitute linkages may be used at R^2-R^5 in oligonucleotides of structure (1). Many substitute linkages are non-ionic and contribute to the desired ability of the oligomer to diffuse across membranes. These "substitute" linkages are defined herein as conventional alternative linkages such as phosphorothioate or phosphoramidate, are synthesized as

described in the generally available literature. Substitute linkages groups thus include, but are not limited to linkages comprising a moiety of the formula -O-P(O)S-O-, ("thioate"), -O-P(S)S-O- ("dithioate"), -O-P(O)N(R^6)$_2$-O-, -O-P(O)R^6-O-, -O-P(O)OR^7-O-, -O-CO-O-, or -O-CON(R^6)$_2$-O- wherein each R^6 independently is H (or a salt) or alkyl (C_{1-8}) and R^7 is alkyl (C_{1-8}). Also included are alkylphosphonate linkages such as methyl-, ethyl- or propylphosphonates. Substitute linkages that may be used in the oligonucleotides disclosed herein also include nonphosphorous-based internucleotide linkages such as the 3'-thioformacetal (-S-CH_2-O-), 5'-thioformacetal (-O-CH_2-S-), formacetal (-O-CH_2-O-), 5' amine (-CH_2-CH_2-NR^{13}- where R^{13} is hydrogen, a protecting group or alkyl C_{1-6}, see, e.g., PCT US91/06855), and 3'-amine (-NR^{13}-CH_2-CH_2-) internucleotide linkages.

Substitute linkages suitable for R^2 or R^2-R^5 and for other invention oligonucleotides have been described, e.g., phosphorodithioates (Marshal, *Science* 259:1564, 1993), phosphorothioates and alkylphosphonates (Kibler-Herzog, *Nucleic Acids Research* 19:2979, 1991; PCT 92/01020; EP 288,163), phosphoroamidates (Froehler, *Nucleic Acids Research* 16:4831, 1988), phosphotriesters (Marcus-Sekura, *Nucleic Acids Research* 15:5749, 1987), boranophosphates (Sood, *J. Am. Chem. Soc.* 112:9000, 1991), 3'-O-5'-S-phosphorothioates (Mag, *Nucleic Acids Research* 19:1437, 1991), 3'-S-5'-O-phosphorothioates (Kyle, *Biochemistry* 31:3012, 1992), 3'-CH_2-5'-O-phosphonates (Heinemann, *Nucleic Acids Research* 19:427, 1991), 3'-NH-5'-O-phosphonates (Mag, *Tet. Lett.* 33:7323, 1992), sulfonates and sulfonamides (Reynolds, *J. Org. Chem.* 57:2983, 1992), sulfones (Huie, *J. Org. Chem.* 57:4519, 1992), sulfoxides (Huang, *J. Org. Chem.* 56:3869, 1991), sulfides (Schneider, *Tet Lett.* 30:335, 1989), sulfamates, ketals and formacetals (Matteucci, *J. Am. Chem. Soc.* 113:7767, 1991, PCT 92/03385 and PCT 90/06110), 3'-thioformacetals (Jones, *J. Org. Chem.* 58:2983, 1993), 5'-S-thioethers (Kawai, *Nucleosides Nucleotides* 10:1485, 1991), carbonates (Gait, *J. Chem. Soc. Perkin Trans 1* 1389, 1979), carbamates (Stirchak, *J. Org. Chem.* 52:4202, 1987), hydroxylamines (Vasseur, *J. Am. Chem. Soc.* 114:4006, 1992), methylamine (methylimines) and methyleneoxy (methylimino) (Debart, *Bioorg. Med. Chem. Lett.* 2:1479, 1992) and amino (PCT 91/06855), hydrazino and siloxane (U.S. Patent 5,214,134) linkages.

Substitute linkages also are known for the replacement of the entire phosphoribosyl group of conventional oligonucleotides. These include for example morpholino-carbamates (Stirchak, *Nucleic Acids Research* 17:6129, 1989) and riboacetal linkages (PCT 92/10793).

Additional substitute linkages suitable for use in the invention oligonucleotide analogs are disclosed in PCT 91/08213, 90/15065, 91/15500, 92/20702, 92/20822, 92/20823, 92/04294, 89/12060 and 91/03680; Mertes, *J. Med. Chem.* 12:154, 1969; Mungall, *J. Org. Chem.* 42:703, 1977; Wang, *Tet Lett* 32:7385, 1991; Stirchak, *Nucleic Acids Research* 17:6129, 1989; Hewitt, *Nucleosides and Nucleotides* 11:1661, 1992; and U.S. Patents 5,034,506 and 5,142,047.

The phosphodiester or substitute linkages herein are used to bond the 2' or 3' carbon atoms of ribose or ribose analogs to the 5' carbon atoms of the adjacent ribose or ribose analog. Ordinarily, the linkages in oligonucleotides are used to bond the 3' atom of the 5' terminal oligonucleotide to the 5' carbon atom of the next 3'-adjacent nucleotide or its analog.

As used herein, "sugar" includes furanose moieties usually found in nucleic acids and their isomers, e.g., arabinose, as well as other sugars, hexoses such as glucose. Sugar also includes carbocyclic analogs of these sugars. Sugars optionally comprise modification of the 2' or 3' position by a O-hydrocarbyl, NH-hydrocarbyl, S-hydrocarbyl group, O-pseudohydrocarbyl, NR^{14}-pseudohydrocarbyl, or S-pseudohydrocarbyl group, including 2'- or 3'-O-methyl, O-ethyl, O-propyl, O-isopropyl, O-butyl, O-isobutyl, O-propenyl or O-allyl, which are used due to their increased lipophilicity compared to the 2'-hydrogen or 2'-hydroxyl found in unmodified DNA or RNA. Corresponding S-alkyl or NH-alkyl substituents may also be utilized. Modifications such as 2'-O-alkyl C_{1-4}, 2'-O-haloalkyl C_{1-4} and 2'-fluoro are generally suitable for binding competent oligonucleotides and may thus be used to prepare the oligonucleotides. One may modify the 2' or 3' position using pseudohydrocarbyl groups. Such groups will typically contain about 1-6 carbon atoms and include substituents such as 2'- or 3'-O-$(CH_2)_{1-3}$-O-$(CH_2)_{1-3}$-R^{11} where R^{11} is a halogen, hydrogen, hydroxyl or NHR^{12} and R^{12} is hydrogen or a protecting group, and substituents such as 2'- or 3'-O-$(CH_2)_{1-2}$-O-$(CH_2)_{1-2}$-O-$(CH_2)_{1-2}$-R^{11}. Workers have described such modifications (see, e.g., U. S. patent numbers 5,466,786 and 5,399,676; PCT US91/03680; Cotten, M., et al., *Nucleic Acids Res* 19:2629-2635 1990; Blencowe, B.J., et al., *Cell* 59:531-539 1989; Sproat, B.S., et al., *Nucleic Acids Res* 17:3373-3386 1989; Inoue, H., et al., *Nucleic Acids Res* 15:6131-6148 1987; Morisawa, H., et al., European Patent Publication No. 0339842; Chavis, C., et al., *J Organic Chem* 47:202-206 1982; Sproat, B.S., et al., *Nucleic Acids Res* 19:733-738 1991). The 2'-modified oligomers were reported to be relatively nuclease stable compared to unmodified controls (Guinosso, C.J., et al., *Nucleosides and Nucleotides*

10:259-262 1991). Synthesis of 2'-fluoro nucleosides and their incorporation into oligonucleotides has also been described (Codington, J.F., et al., *J Org Chem* 29:558-564 1964; Fazakerley, G.V., et al., *FEBS Lett* 182:365-369 1985). Synthesis of oligonucleotide analogs containing the modified bases described herein would be based on methods described. Synthesis of 2'-thioalkyl nucleosides is accomplished as described in U.S. Patent No. 5,484,908.

As used herein "oligonucleotide" or "oligomer" is generic to polydeoxyribonucleotides (containing 2'-deoxy-D-ribose or modified forms thereof such as arabinose or carbocyclic analogs of ribose), i.e., DNA, to polyribonucleotides (containing D-ribose or modified forms thereof), i.e., RNA, and to any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base. Oligonucleotide or oligomer, as used herein, is intended to include (i) compounds that have one or more furanose moieties that are replaced by furanose derivatives or by any structure, usually cyclic, that may be used as a point of covalent attachment for the base moiety, (ii) compounds that have one or more phosphodiester linkages that are either modified, as in the case of phosphoramidate or thioate linkages, or where the phosphorus atom is completely replaced by a suitable linking moiety as in the case of, e.g., formacetal linkages, and/or (iii) compounds that have one or more bonded furanose-phosphodiester linkage moieties replaced by any structure, cyclic or acyclic, that may be used as a point of covalent attachment for the base. Invention oligonucleotides are of any convenient length and generally comprise 2-50 bonded monomers, often 3-20, usually 4-15. Oligonucleotide also includes short molecules such as dimers, trimers and tetramers having a Log value of the octanol:water partition coefficient of -0.3 to +2.5 and a solubility in water of at least 0.001 μg/mL, which are useful as synthetic intermediates. A standard oligonucleotide dimer with two linkage groups has a molecular weight of about 650 Daltons. The corresponding invention oligonucleotides optionally have molecular weights of 1,500 or more, e.g., 1,500-6,000, or 6,000-10,000.

As used herein, the terms "lipophilic oligonucleotide," "lipophilic linkage," lipophilic base," lipophilic sugar," "lipophilic modification," and "lipophilic substitution" mean an oligonucleotide, or a linkage, base, sugar, modification or substitution, respectively, that makes the modified or substituted molecule more lipophilic than the corresponding unmodified molecule at pH 7.4 in water or low ionic strength buffer. An unmodified oligonucleotide means one that is composed of ribose, or 2'-deoxyribose,

phosphodiester linkages and the bases guanine, adenine, cytosine , thymine and/or uracil, i.e., DNA or mRNA.

In general, each modified linkage, base or sugar optionally comprises a single lipophilic modification, although more than one may be present, particularly at a linkage or base. A linkage such as -O-CH_2-O- has a single lipophilic modification, i.e., -CH_2- replaces -P(O)(OH)- in the O-P(O)(OH)-O- linkage. A linkage such as -CH_2-CH_2-CH_2- would have 3 lipophilic modifications compared to the normal phosphodiester linkage in DNA or RNA. However, as used herein, a -CH_2-CH_2-CH_2- linkage is considered to be a single modification for the purpose of determining the % of modifications in an oligonucleotide. Thus, an oligonucleotide having 6 bonded monomers with only -CH_2-CH_2-CH_2- linkages would have 100% of the linkages modified and not 300%. An invention oligonucleotide, such as a structure (1) oligonucleotide, containing modifications at each linkage, base and sugar would have modifications that sum to 300%, i.e., 100% of linkages plus 100% of sugars plus 100% of bases.

Oligonucleotide uptake into cells. We have discovered lipophilic oligonucleotide analogs that enter cells by passive diffusion across cell, endosome and/or organelle membranes. The oligonucleotide analogs may enter cells by multiple mechanisms, e.g., pinocytosis, receptor-mediated uptake, phagocytosis as well as by passive diffusion. It has been generally assumed that oligomers containing the native phosphodiester linkages enter cells by receptor-mediated endocytosis (Loke, S.L., et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 86:3474-3478 1989; Yakubov, L.A., et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 86:6454-6458 1989). Subsequent studies appear to show that oligomers with modified internucleotide linkages that may mitigate the presence of negative charges also enter the cells through specific receptors, rather than by passive diffusion (Akhtar, S., et al., *Nucl. Acids Res.* 19:5551-5559 1991; Shoji, Y., et al., *Nucl. Acids Res.* 19:5543-5550 1991). Entry of oligomers into cells by either receptor mediated endocytosis or by other mechanisms results in their localization into intracellular endosomes or vesicles. Thus, entry of oligomers into cellular cytoplasm or nucleoplasm is prevented by the membrane barrier surrounding these subcellular organelles. Because of the low rate of such endocytosis, it has been necessary to attempt to protect the oligonucleotides from degradation in the bloodstream either by inclusion of these materials in protective transport complexes, for example with LDL or HDL (deSmidt, P., et al., *Nucl. Acids Res.* 19:4695-4700 1991) or by capping them with nuclease-

resistant internucleotide linkages (Hoke, G.D., et al., *Nucl. Acids Res.* 19:5743-5748 1991).

No clearly documented progress has been reported in designing oligonucleotides which are capable of passive cell membrane diffusion and enter cells rapidly across cellular membranes to interact with intracellular targets. Those factors related to molecular characteristics which determine the diffusion coefficients of molecules in general have, however, been extensively studied. See, for example, Stein, W.D., in "New Comprehensive Biochemistry", Vol. 2 (Membrane Transport), Elsevier/North Holland Biomedical Press (1981), pp. 1-28; Lieb, W.R., et al., *Nature* 224:240-243 1969. It has been concluded that the distribution constant for a particular substance between the lipophilic membrane and an external aqueous phase is a direct function of the partition coefficient of the material between octanol and water times the molecular weight of the material of interest raised to an appropriate negative power characteristic of the membrane. As the appropriate negative power for, for example, red blood cells is about -4, it appears that high molecular weight substances must have hopelessly low distribution coefficients between cellular membrane and the external environment, even if their partition coefficients for octanol:water are quite high. The validity of this relationship for various small molecules, however, appears to be substantiated by experiment (Hansch, C., et al., *J. Pharm. Sci.* 61:1-19 1972; Walter, A., et al., *J. Membrane Biol.* 90:207-217 1986).

The partition coefficient for native DNA or RNA is relatively low with a log value of the octanol:water partition coefficient being less than -4 (Dagle, J.M., et al., *Nucl. Acids Res.* 19:1805-1810 1991). DNA modified by synthesis of 2-methoxyethylphosphoramidite internucleoside linkages in place of the phosphodiester linkage eliminates the negative charge associated with the internucleotide linkage, which increases the hydrophobicity of DNA. However, the log value of the octanol:water partition coefficient (Log Poct) remains less than -2 (Dagle, supra). Increased Log Poct values for 2-methoxyethylphosphoramidite-modified DNA were assayed by measuring the partitioning of radiolabeled DNA in an octanol-aqueous buffer system. Increased Log Poct was correlated with increased retention time on reversed-phase HPLC columns (Dagle, supra). Other DNA analogs, such as methylphosphonates or phosphorothioates, or DNA with lipophilic adducts (Severin, E.S., et al., *Adv. Enzyme Regulation* 31:417-430 1991) that are described in the literature are similarly expected to have Log Poct values less than 0.0.

We have found that oligonucleotides can be modified by appropriate design of their molecular features so as to permit their passive diffusion across cellular membranes, despite the high molecular weights inherent in these molecules. Because of the high molecular weights of the invention oligonucleotide analogs, the relevant factor generated in determining distribution between membrane and aqueous medium is very small, which indicates that such a molecule is essentially impermeable to cell membranes. Despite this, the dimers and higher molecular weight oligonucleotides of this invention are, however, capable of passive diffusion into cells, and are thus not sequestered exclusively in endosomes in the same manner as described for previously known oligonucleotide analogs (Fisher et al., *Nucl. Acids Res.* 21:3857-3865 1993).

An aspect of the invention oligonucleotide analogs is our finding that when one introduces lipophilic modifications into multiple sites on the oligonucleotide, the oligonucleotide usually will passively diffuse into cells. We believe this may be due, at least in part, to the use of multiple, relatively small lipophilic modifications, such as hydrocarbyl or pseudohydrocarbyl moieties containing about 1-8 carbon atoms, at several locations on the oligonucleotide, rather than one or several large lipophilic moieties such as cholesteryl groups (see, e.g., Kabanov et al., *FEBS* 259:327-330 1990, Severin et al., *Adv. Enz. Regul.* 31:417-430 1991, EP 0 462 145 B1, U. S. Patent Nos. 4,958,013 and 5,420,330). The modifications of this invention that render an oligonucleotide analog permeation competent and soluble in aqueous media, as hereinbelow defined, are thus located at more than one location on the oligonucleotide which alters its overall lipophilicity character, making the molecule capable of efficient partitioning across membranes.

Oligonucleotide synthesis. Oligonucleotides and the nucleotide synthons therefor are conventionally synthesized. Methods for such synthesis are found, for example, in Froehler, B., et al., *Nucleic Acids Res.* 14:5399-5467 1986; *Nucleic Acids Res.* 16:4831-4839 1988; *Nucleosides and Nucleotides* 6:287-291 1987; Froehler, B., *Tetrahedron Letters* 27:5575-5578 1986. Amine, carboxyl and hydroxyl groups present anywhere on the molecule may be protected during oligonucleotide synthesis using standard protecting groups. Other conventional methods may be used to synthesize the oligomers or segments thereof, including methods employing phosphoramidite chemistry and/or methods that utilize solution phase synthesis. For oligonucleotide synthesis, R or R^1 optionally are oligonucleotide coupling groups. "Coupling

group" as used herein means any group suitable for generating a linkage or phosphodiester substitute linkage between nucleotide bases or their analogs. These coupling groups are conventional and well-known for the preparation of oligonucleotides, and are prepared and used in the same fashion here. In general, each compound of structure (1) will contain two blocking groups: R or R^1, but with only one of them being a coupling group. The coupling groups are used as intermediates in the preparation of 3'-5' 5'-3', 5'-2' and 2'-5' internucleotide linkages in accord with known methods.

As used herein, the "blocking group" of R or R^1 refers to a substituent other than OH that is conventionally coupled to oligomers or nucleosides, either as a protecting group, an activated group for synthesis or other conventional conjugate partner such as a solid support, label, immunological carrier and the like. Suitable protecting groups are, for example, hydroxyl protecting groups such as DMT, MMT or FMOC; suitable activated groups are, for example, H-phosphonate, methyl phosphonate, methylphosphoramidite or β-cyanoethylphosphoramidite. R or R^1 may also comprise a solid support. In general, the nucleosides and oligomers of the invention may be derivatized to such "blocking groups" as indicated in the relevant formulas.

Suitable coupling groups for phosphodiester linkages include OH, H-phosphonate; (for amidite chemistries) alkylphosphonamidites or phosphoramidites such as beta-cyanoethylphosphoramidite, N, N-diisopropylamino-beta-cyanoethoxyphosphine, N,N-diisopropylamino-methoxyphosphine, N,N-diethylamino-methoxyphosphine, N,N-diethylamino-beta-cyanoethoxyphosphine, N-morpholino-beta-cyanoethoxyphosphine, N-morpholino methoxyphosphine, bis-morpholino-phosphine, N,N-dimethylamino-beta-cyanoethylmercapto-phosphine, N,N-dimethylamino-2,4-dichlorobenzylmercapto-phosphine, and bis(N,N-diisopropylamino)-phosphine; and (for triester chemistries) 2-, or 4-chlorophenyl phosphate, 2,4-dichlorophenyl phosphate, or 2,4-dibromophenyl phosphate. See for example U.S. patents 4,725,677; 4,415,732; 4,458,066; and 4,959,463; and PCT 92/07864. If R^1 is a coupling group then R typically will be hydroxyl blocked with a group suitable for ensuring that the monomer is added to the oligomer rather than dimerizing. Such groups are well known and include DMT, MMT, FMOC (9-fluorenylmethoxycarbonyl), PAC (phenoxyacetyl), a silyl ether such as TBDMS (t-butyldiphenylsilyl) and TMS (trimethylsilyl). Obviously, the opposite will apply when one desires to synthesize an oligomer in the opposite direction (5'→3'). Ordinarily, R is

DMT, R^1 is located on the 3' carbon, the remaining R^1 is H and the R^1 groups are in the alpha anomer conformation.

In addition to solid phase synthesis techniques, oligonucleotides may also be partially or fully synthesized using solution phase methods such as triester synthesis. These methods are workable, but in general, less efficient for oligonucleotides of any substantial length.

Linkage synthesis. Table A below sets forth various examples of suitable substitute linkages for use with the oligonucleotide analogs of this invention. The columns designated R^A (5') and R^B (3' or 2') describe substituents used to produce the R^2-R^5 linkage of structure (1), shown in the right column, using methods known per se in the art and described in PCT US93/05202 and other citations above. The starting materials in Table A, or those used to prepare the starting materials of Table A, generally possess a ribose or a ribose analog comprising a 5' hydroxyl group and a 3' or 2' hydroxyl group, prepared as described herein or in the citations, with the substitute linkage being substituted for the phosphodiester linkages in unmodified nucleic acids. Sequentially useful starting materials are designated by an arrow. Bracketed monomers are reacted to form dinucleotide analogs having the R^2-R^5 substitute linkage. The reactions are repeated or ganged with phosphodiester linkages in order to produce trimers, tetramers or larger oligomers.

Bl in Table A means a blocking group. As used herein, "blocking group" refers to a substituent other than H that is conventionally attached to oligomers or nucleotide monomers, either as a protecting group, a coupling group for synthesis, PO_3^{-2}, or other conventional conjugate such as a solid support. As used herein, "blocking group" is not intended to be construed solely as a nucleotide protecting group, but also includes, for example, coupling groups such as hydrogen phosphonate, phosphoramidite and others as set forth herein. Accordingly, blocking groups are species of the genus of "protecting groups" which as used herein means any group capable of preventing the O-atom or N-atom to which it is attached from participating in a reaction involving an intermediate compound of structure (1) or otherwise forming an undesired covalent bond. Such protecting groups for O- and N-atoms in nucleotide monomers or nucleoside monomers are described and methods for their introduction are conventionally known in the art. Protecting groups also are useful to prevent reactions and bonding at

carboxylic acids, thiols and the like as will be appreciated by those skilled in the art.

Table A

Substitute Linkages

R (5')	R^A (3' or 2')	2'/3'-R^B-5'
OH→DMTO NH_2	$-CH_2CH=CH_2 \rightarrow CH_2CHO$ OBl	$-(CH_2)_2-NHCH_2-$
OH→DMTO $CH_2C(OEt)_2$	$N_3 \rightarrow NH_2$ -OBl	$-NH(CH_2)_2-$
OH→DMTO $-CH_2NH_2$	$-CH_2CH=CH_2 \rightarrow CH_2CHO-$ -OBl	$-CH_2NH(CH_2)_2-$
OH→DMTO $-CH_2NH_2$	OH→$-OCH_2CH=CH_2$ -OBl	$-O(CH_2)_2NHCH_2-$
OH→$-OCH_2CH=CH_2$ OH→DMTO	OBl NH_2	$-NH(CH_2)_2OCH_2-$
DMTO $-NH_2$	CHO OBl	$-CH_2NHCH_2-$
$CH_2CN \rightarrow CH_2$ CHO DMTO	OBl NH_2	$-NH(CH_2)_2-$
$(CH_2)_2OH$→ $(CH_2)_2OTs$ DMTO	OBl SH	$-S(CH_2)_3-$; $-S(O)(CH_2)_3-$; or $-S(O)(O)(CH_2)_3-$
$CH_2OH \rightarrow CH_2Br$ DMTO	OBl SH	$-S(CH_2)_2-$; $-S(O)(CH_2)_2-$; or $-S(O)(O)(CH_2)_2-$
DMTO SH	$CH_2O \rightarrow CH_2OH \rightarrow CH_2OTS$ OBl	$-CH_2SCH_2-$; $-CH_2S(O)CH_2-$; or $-CH_2S(O)(O)CH_2-$
$TsOCH_2$ DMTO	OBl OH	$-O(CH_2)_2-$

R (5')	R^A (3' or 2')		$2'/3'-R^B-5'$
DMTO	$CH_2CHO \rightarrow (CH_2)_2OH$	}	$-(CH_2)_2OCH_2-$
OH → MsO	OBl	}	
DMTO	NHalk(C1-6)	}	$-N(alk)(CH_2)_3-$
CH_2CHO	OBl	}	
DMTO	NH(COOEt) →	}	
	$N(COOEt)(CH_2SCH_3)$	}	$-N(COOEt)CH_2OCH_2-$
OH	OBl	}	
$(CH_2)_2I$	OBl	}	$-S(CH_2)_3-$
DMTO	SH	}	
TolO	NH_2	}	$-NHC(O)OCH_2-$
pNPhOC(O)O	OBl	}	
TolO	OCH_2Cl	}	$-OCH_2SCH_2-$
SH	OBl	}	
TolO	OC(O)OpNPh	}	$-OC(O)N(R)CH_2-$
-NHR (R=H or lower alkyl)	OBl	}	
TolO	OCH_2SMe	}	$-OCH_2OCH_2-$
OH	OBl	}	
DMTO	SH	}	$-SCH_2OCH_2-$
OCH_2Cl	OBl	}	
DMTO	OH	}	$-OCH_2CH=CH-$
$BrCH_2CH=$	-OBl	}	
DMTO	SH	}	$-SCH_2CH=CH-$
$BrCH_2CH=$	-OBl	}	

Invention embodiments. The invention oligonucleotides are characterized by a log value of the partition coefficient between octanol and water of about -0.3 to +2.5 and a solubility in water of at least 0.001 μg/mL, usually at least 0.01 μg/mL. In general, the invention oligonucleotides will have at least 60%, often at least 80%, of their internucleotide linkages as lipophilic modifications or at least 60%, often at least 80%, of their bases will contain a lipophilic substitution, or at least 60%, often at least 80%, of their

sugars will contain a lipophilic substitution, or wherein the percent non-ionic nucleotide linkages, the percent bases containing a lipophilic substitution and the percent sugars containing a lipophilic substitution sums to at least 60%, often at least 80%. The linkages in the invention oligonucleotides are present as ionic linkages, non-ionic linkages or as a mixture of ionic and non-ionic linkages. Lipophilic modifications for the oligonucleotides are independently chosen for each modification of the molecule. Generally, the log value of the partition coefficient between octanol and water will be 0.0-2.5, typically 0.2-2.3, usually 0.6-2.1.

Invention embodiments include structure (1) oligonucleotide analogs wherein the linker at R, R^1 or R^3 optionally has 1-10 carbon, oxygen, sulfur and/or nitrogen atoms bonded together in an uninterrupted chain. The linker, which may be bonded to any invention oligonucleotide analog, including structure (1) oligonucleotides, will either connect the oligonucleotide analog to a detectable moiety or is bonded to the oligonucleotide analog at one end of the linker and, at the other end, has a reactive group available for bonding with a detectable moiety, e.g., amine, carboxyl or hydroxyl group. The carbon or other atoms that comprise the uninterrupted linker chain may be substituted with one or more substituents that do not interfere with the function of the linker or the oligonucleotide analog, e.g., hydrocarbyl containing 1, 2, 3, 4, 5 or 6 carbon atoms or pseudohydrocarbyl containing 1, 2, 3, 4, 5 or 6 carbon atoms and one or more of the substitutions described above. Invention oligonucleotide analogs, such as structure (1) oligonucleotides optionally have no linkers, but when a linker is present there is usually only 1 linker which, for structure (1) is located at R, R^1, R^3 or a base, e.g., at the 5 position of pyrimidines. Occasionally, the oligonucleotides, including structure (1) oligonucleotides, contain 2, 3 or more linkers, which are usually linkers having 1-10 bonded atoms that form an uninterrupted chain.

For structure (1) oligonucleotides, each R^2 independently is a phosphodiester linkage or another linkage bonded to the 2' or 3' position and usually any linkage containing a phosphorus atom is bonded to the 3' position. For structure (1) oligonucleotides, each R^3 independently is in the ribo or ara configuration and independently is H, OH, F, blocked hydroxyl, $N(R^{14})_2$, O-hydrocarbyl including -O-alkyl (C_{1-8}) and -O-alkenyl (C_{3-8}), and O-pseudohydrocarbyl including -O-alkyl (C_{1-8}) where the alkyl group is substituted with halogen, hydroxyl, NO_2, N_3, carboxyl, ester, amide or oxygen as a keto or ether moiety, -S-alkyl (C_{1-8}) or a linker, wherein the linker

optionally has 1-10 carbon atoms, usually each R^3 independently is H or -O-alkyl (C_{1-3}). When R^3 is in the ara configuration it is usually H or F. Usually R^4 is O. Usually R^5 is CH_2. Usually R^6 is H or CH_3. Generally one R^{14} is hydrogen, C_{1-6} hydrocarbyl or C_{1-8} pseudohydrocarbyl, and usually the other R^{14} is hydrogen or a protecting group. Usually n is 2-13.

In invention oligonucleotide analogs, including structure (1) oligonucleotides, each B independently is a base or an analog thereof in the α or β anomeric configuration, usually in the β configuration, wherein base analogs are optionally lipophilic analogs that comprise a C_{1-8} alkyl, C_{5-8} cycloalkyl, C_{6-8} aryl, C_{3-8} heteroaryl, C_{2-8} alkenyl or C_{2-8} alkynyl moiety or the analog contains a linker having 1-10 carbon atoms, wherein the oligonucleotide has a log value of the octanol:water partition coefficient of about -0.3 to +2.5, generally 0.0-2.5, typically 0.2-2.3, usually 0.6-2.1 and having a solubility in water of at least 0.001 μg/mL, usually at least 0.01 μg/mL. Oligonucleotides having nucleosides containing bases in the α anomeric configuration binds to duplexes in a manner similar to that for the β anomers, and one or more nucleosides may contain a base in the α anomeric configuration, or more typically a domain thereof. (Praseuth, D., et al., *Proc Natl Acad Sci (USA)* 85:1349-1353 1988).

For invention oligonucleotide analogs, or for structure (1) oligonucleotides, the linkage (R^2-R^5) will generally be a linkage moiety that is 3-5 atoms in length, usually 4. The linkages typically comprise bonded carbon, oxygen, nitrogen, sulfur and/or phosphorus atoms in an uninterrupted chain.

Invention embodiments include oligonucleotide analogs of structure (1) wherein each lipophilic substitution at substituted bases independently is a hydrocarbyl group, typically a C_{1-8} hydrocarbyl group or a pseudohydrocarbyl group that is substituted with one or more heteroatoms selected from the group consisting of nitrogen, oxygen and sulfur, typically a C_{1-8} pseudohydrocarbyl group. Exemplary lipophilic substitutions include ones wherein the C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl group is bonded to a purine or pyrimidine base position selected from the group consisting of a C5 position of pyrimidines, the O4 position of thymine, the N6 position of adenine, the C8 position of adenine, the N2 position of guanine, the C8 position of guanine, the N4 position of cytosine and the C7 position of 7-deazapurines.

Any phosphate present at the 5' or 3' terminus of the oligonucleotide is optionally derivatized, for example, by further esterification to a lipophilic

group containing about 3-8 carbon atoms. A particularly useful derivatizing group may contain a linker and a label, for example, a fluorescent label such as fluorescein, rhodamine, or dansyl. Thus, useful derivatizing groups include Fl-CONH $(CH_2)_{2-8}$- and Rh-CONH $(CH_2)_{2-8}$-, wherein Fl and Rh signify fluorescein and rhodamine, respectively.

Invention oligonucleotides are optionally coupled to a label such as a fluorescent moiety and usually the label is coupled through a linker. A linker is a moiety that contains an uninterrupted chain of atoms, e.g., 1-25 bonded (or linked) atoms, that connect the label and the oligonucleotide analog. Generally the linker contains 1-10 bonded atoms in such a chain. The invention oligonucleotides are optionally able to bind single or double-stranded nucleic acid in a sequence-specific manner when the lipophilic base, sugar and linkage modifications do not completely destroy the oligonucleotide's binding capacity. Such modifications are known and include, e.g., (a) an alkynyl group such as 1-propynyl or 1-butynyl present at the 5 position of pyrimidine bases or pyrimidine base analogs, (b) an alkynyl group such as 1-propynyl or 1-butynyl present at the 7 position of 7-deazapurine bases or 7-deazapurine base analogs, (c) non-ionic linkages such as formacetal linkages or (d) sugar analogs where R^3 is -O-alkyl (C_{1-3}) or F.

One introduces the invention oligonucleotide analogs into cells by contacting the cells an invention oligonucleotide. The oligonucleotide may be delivered into cells in tissue culture medium containing serum or lacking serum, or the oligonucleotide may be in an aqueous buffer or solution such as PBS. One uses this method to deliver invention oligonucleotides into cells *in vitro* or *in vivo*.

Invention embodiments include a method to visualize cells, usually viable cells which are optionally mammalian cells in tissue culture or *in vivo*, which method comprises: (a) contacting the cells to be visualized with an invention oligonucleotide containing a detectable moiety, e.g., a fluorescent moiety or radioactive moiety, under conditions wherein passive diffusion across the cell membrane can occur so as to internalize the oligonucleotide; (b) washing the cells to remove any oligonucleotide which has not passively diffused across the membrane and become internalized; (c) and detecting the internalized oligonucleotide to visualize the cells. One optionally performs the method using an invention oligonucleotide having a solubility of at least about 10 μM, usually at least 50 μM in water, to facilitate visualizing the stained cells using fluorescence methods. The inventors have found that some of the invention oligonucleotides are suitable for staining or visualizing

a subcellular compartment of the viable cell. One can stain or visualize subcellular compartments including endoplasmic reticulum, nuclear envelope, cell nuclei and mitochondria.

Using oligonucleotides of structure (1) as a reference, when at least 80% of the linkages are modified, trimers have 2 modified internucleotide linkages and tetramers have 3, and so forth. Thus, all internucleotide linkages must be converted to lipophilic forms for oligomers which are less than hexamers. For hexamers, having 5 internucleotide linkages, only 4 of these need to be modified if only linkages, but not any bases or sugars, are modified.

One optionally includes in the invention oligonucleotide analogs relatively large lipophilic moieties, e.g., 1, 2, 3 or 4 C_{9-16} hydrocarbyl substituents, or 1, 2, 3, or 4 C_{14-18} pseudohydrocarbyl substituents. However, such oligonucleotide analogs will generally contain either (1) a domain or structural feature in the large hydrocarbyl or pseudohydrocarbyl substituent that limits membrane binding, e.g., branched, polar or charged groups or regions or (2) there will usually only be 1 or 2 of such large groups, if they are compatible with membrane binding. Usually, such large hydrocarbyl or pseudohydrocarbyl groups, particularly if they are compatible with membrane binding, will not be located on adjacent monomers, i.e., there will be 1, 2, 3 or more monomers that contain a smaller lipophilic group or no lipophilic group interspersed between the monomers containing the larger groups.

To possess the desired diffusion properties, the invention oligonucleotide analogs will generally contain a lipophilic modification at least at 60%, often at least 80%, of either their internucleotide linkages, sugars and/or their bases. Invention embodiments include oligonucleotide analogs where no sugar contains a lipophilic modification and at least 60%, often at least 80%, of the internucleotide linkages are non-ionic or at least 60%, often at least 80%, of the bases contain a lipophilic modification. Other embodiments include oligonucleotide analogs where no base contains a lipophilic modification and at least 60%, often at least 80%, of the internucleotide linkages are non-ionic or at least 60%, often at least 80%, of the sugars contain a lipophilic modification. Other embodiments include oligonucleotide analogs where no linkage is non-ionic and at least 60%, often at least 80%, of the bases contain a lipophilic modification or at least 60%, often at least 80%, of the sugars contain a lipophilic modification. Thus, for a 10-mer oligonucleotide, the oligonucleotide will have 6 modified or substituted linkages, bases or sugars, or two each may be modified or substituted or the

oligonucleotide will have some combination that results in 6 modifications or substitutions at the linkages, sugars or bases.

Larger hydrocarbyl groups, e.g., ones containing 9-18 carbon atoms will generally contribute more lipophilic character to a nucleoside, i.e., a base, sugar or linkage, than is required for permeation competence. In addition, the presence of several of the larger groups such as ones containing 16-20 carbon atoms may result in anchoring of the oligonucleotide in cell membranes. One will thus generally use C_{1-8} hydrocarbyl groups and/or C_{1-8} pseudohydrocarbyl groups to obtain a sufficiently lipophilic monomer. For example, replacement of the -P(O)(O^-)- group in a phosphodiester linkage with the hydrocarbyl moiety -CH_2- confers a significant amount of lipophilic character on the linkage, due to the loss of the negative charge associated with the phosphodiester moiety. The use of C_{1-8} hydrocarbyl groups generally confers sufficient non-ionic character and lipophilicity on the oligonucleotide to result in a compound that conforms to the required solubility and distribution coefficient values. One confirms this by assays.

By the use of appropriate larger lipophilic substituents, such as moieties containing about 10-16 carbon atoms, the proportion of either bases, sugars and/or linkages that must be modified for permeation competence can be reduced to about 40-60% or less. When designing such oligonucleotides, one will generally use at least one structure such as a branched alkyl group or pseudohydrocarbyl group having a polar moiety (e.g., a hydroxyl group) or a polar domain to decrease the tendency of the lipophilic substituent to anchor the oligonucleotide in a membrane.

Invention oligonucleotides have a log value of the partition coefficient between octanol and water of -0.3 to +2.5, generally 0.0-2.5, typically 0.2-2.3, usually 0.6-2.1. These oligonucleotides optionally contain C_{1-8} hydrocarbyl substituents, generally C_{1-6} hydrocarbyl substituents, or C_{1-14} pseudohydrocarbyl substituents, generally C_{2-8} psudohydrocarbyl substituents. Such oligonucleotides optionally contain 1 or 2 C_{9-12} hydrocarbyl substituents or they optionally contain 1 or 2 C_{15-18} pseudohydrocarbyl substituents. Such oligonucleotides typically contain 3-30 monomers, usually 4-15. The hydrocarbyl or pseudohydrocarbyl substituents are generally located at least at 60%, usually at least at 80% of the linkages, bases or sugars, or the oligonucleotides have these substituents at a combination of the linkages, bases and sugars that sums to at least 60% or at least 80%. Exemplary embodiments include oligonucleotides wherein the hydrocarbyl or pseudohydrocarbyl substituents are located on 60-90%, usually at 80-120% of

the linkages, bases or sugars. Alternatively, the oligonucleotides have these substituents on a combination of the linkages, bases and sugars that sum to 60-90% or 80-120%. Exemplary embodiments include oligonucleotides having branched or unbranched alkyl, alkenyl or alkynyl hydrocarbyl groups (C_{1-8}) or branched or unbranched alkyl, alkenyl or alkynyl pseudohydrocarbyl groups (C_{1-14}) at the linkages, bases and/or sugars. Other embodiments include oligonucleotides having cyclic alkyl or alkenyl hydrocarbyl groups (C_{5-8}) or cyclic alkyl or alkenyl pseudohydrocarbyl groups (C_{3-8}) at the linkages, bases and/or sugars.

In some embodiments, it may be desirable to substitute oligonucleotides of the invention with base, linkage and/or sugar modifications that do not significantly interfere with the capacity of the oligonucleotide to bind to a complementary nucleic acid target. The target may be a single-chain or duplex nucleic acid. Appropriate substitutions for binding competent modified oligomers with target nucleic acids refer to substitutions at base, linkage and/or sugar positions that do not completely disrupt the oligonucleotide's capacity to hydrogen bond with complementary nucleic acids. Those positions on bases include the N6 or C8 of adenine, the N2 or C8 of guanine, the C5 of pyrimidines, N4 of cytosine and C7 of 7-deazapurines. Synthesis of such modified bases is described in the art, as are methods for incorporation of such bases into oligonucleotides by solid-phase or solution-phase methods (Uhlmann, E., et al., *Chemical Reviews*, 90:543-584 1990, and references cited therein, U. S. Patent No. 5,484,908, PCT US91/06855 and PCT US92/09195). Thus, for invention oligonucleotides designed to bind single-stranded or double-stranded nucleic acid targets, care must be taken to place the lipophilic substituents in such a way so as to avoid disruption of binding to the target. However, for uses of the invention oligonucleotide analogs that do not involve binding to complementary sequences by base pairing, e.g., staining cells or subcellular components, the bases, sugars or linkages may have modifications that are not compatible with oligonucleotide binding competence or with base pairing.

Representative lipophilic substituents at the base residues include saturated and unsaturated straight-chain, branched-chain, or cyclic hydrocarbyl groups, such as an alkane C_{1-8} (usually C_{2-4}), alkene C_{1-8} (usually C_{2-4}), or an alkyne C_{1-8} (usually C_{2-4}), including ethynyl, vinyl, isopropyl, isobutyl, butynyl, butenyl, pentyl, pentenyl, isopentyl, phenethyl, methyl, ethyl, propyl, propynyl, phenyl, phenylvinyl, propenyl, butyl, pentynyl and their

stereoisomers and positional isomers substituted at appropriate positions on the base.

Exemplary base substituted nucleosides include 5-ethynyl-dU (5-ethynyl-2'-deoxyuridine), 5-ethynyl-dC, 8-ethynyl-dG, 5-vinyl-dU, 5-ethyl-dU, 8-ethynyl-dA, 8-propynyl-dG, 8-propynyl-dA, 5-pentyl-dU, 5-pentynyl-dU, 5-phenethyl-dU, 5-pentyl-U, 5-pentynyl-U, 5-benzyl-dC, N6-methyl-8-oxo-2'-deoxy-A, 4-O-butyl-T, 5-propynyl-dC and 5-propynyl-dU.

Invention oligonucleotide analogs are optionally labeled using a detectable moiety, such as a fluorescent label, radiolabel or enzyme label. One links detectable moieties to the oligonucleotide analog using a linker. A wide range of linkers are known and may be used by known methods. Exemplary linkers include linkers that having 2-10 carbon atoms and contain reactive groups that are convenient for linking to oligonucleotides, e.g., amines, hydroxyls, thiols or carboxylic acids. Exemplary linkers include ones having a structure such as H_2N-$(CH_2)_{2-10}$-NH_2 or H_2N-$(CH_2)_{2-5}$-R^5-$(CH_2)_{2-5}$-NH_2, where R^5 is O, C(O), NR^{10}, S, SO or SO_2 where R^{10} is hydrogen, alkyl C_{1-4} or a protecting group. Such linkers would link an invention oligonucleotide, at e.g., the R, R^1 or R^3 position of structure (1) oligonucleotides, to a detectable moiety. Many linkers are available commercially. Linkers can also comprise a chelating agent that binds to a detectable atom (see, e.g., U.S. Patent No. 5,534,497).

Therapeutic methods which utilize oligonucleotides as active agents are based on a number of end strategies. One method, the "antisense" approach wherein the oligonucleotide is designed to be the antisense counterpart of an mRNA transcript and is thus expected to interrupt translation of a gene which has an undesired effect in the cell. More recently, it has been found possible to utilize the polymerase chain reaction (PCR) to amplify selectively oligonucleotides that empirically preferentially bind to targets of diverse molecular structure, including proteins and lipids (see, e.g., PCT US92/01383). The antisense approach permits targeting of any desired nucleic acid sequence, e.g., mRNA, by the properly selected oligonucleotide. The ability to obtain specifically binding oligonucleotides in this way has expanded the possibilities for oligonucleotide therapy because one can design oligonucleotides to target substances that reside at the cellular surface or at intracellular locations such as in the cytoplasm or nucleus.

Numerous publications have appeared that describe inhibition of gene expression by exogenously added oligomers in various cell types (Agrawal, S., et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 85:7079-7083 1988; Uhlmann, E., et al.,

Chem. Revs. 90:583-584 1990). However, oligomers added directly to cells enter the cellular cytoplasm at a low efficiency, at best. Many of the apparent sequence-specific effects that have been described are likely to be due to effects on cellular activity that do not arise from binding of the oligomer to target nucleic acid sequences in cytoplasm or nucleoplasm. Gene specific effects do appear to occur by binding of the oligomer to the target sequence for RNA antisense sequences generated in situ that are complementary to a target sequence or in cell-free *in vitro* systems with exogenously added oligomers (Oeller, P.W., et al., *Science* 254:437-439 1991; Joshi, S., et al., *J. Virol.* 65:5524-5530 1991; Haeuptle, M-T., et al., *Nucl. Acids Res.* 14:1427-1448 1986).

The oligonucleotides of the invention, especially when fluorescently labeled and utilized to visualize cells or subcellular structures, are characterized by having a minimum solubility in water or aqueous media of at least 10 nM, usually 50 nM. The minimum solubility requirement is based on the minimum concentration of fluor required by current fluorescent microscopes for visualizing the label. The oligonucleotides of the invention, when utilized as (i) diagnostic or therapeutic agents that bind to intracellular or extracellular structures such as proteins or nucleic acids, or (ii) labeled compounds to detect or visualize complementary nucleic acid sequences, or cells, cell membranes or subcellular components in tissue samples, intact cells or in cell lysates or fractions, are characterized by a minimum solubility in water or aqueous media of at least about 0.001 μg/mL.

Some of the oligonucleotides of the invention were found to bind to specific subcellular components such as endoplasmic reticulum or mitochondria. Because of this, permeation-competent oligonucleotides that are fluorescently labeled can be used to directly visualize live cells or cell components in cell lysates. The aspects of the compounds that confer subcellular component-specific binding on the oligonucleotides of the invention are believed not to reside in the fluorescent moiety that is attached to the compound. However, the same oligonucleotides, either containing the fluorescent label or without the label can be synthesized utilizing, say, ^{32}P or ^{14}C instead of the normal nonradioactive phosphorus or carbon isotope. Any other appropriate radiolabel can also be utilized according to conventional methods. Such radiolabeled oligonucleotides would retain their cell component-specific binding properties, but need not be directly visualized. In this case, cells or cell lysates can be specifically bound by the oligonucleotide followed by detection of bound oligonucleotide as a means to measure the presence or amount of bound material. Radiolabeled oligonucleotides used in

this manner would have a minimum solubility requirement in water or aqueous media of about 0.001 μg/mL in order to be conveniently detected or quantitated by conventional methods such as scintillation counting.

The distribution coefficient need not be determined directly; that is, the distribution of the material obtained by mixing it with octanol and water and then effecting equilibrium distribution need not be evaluated, see e.g., Dagle et al., *Nucl Acids Res.* 19:1805-1810 1991. Alternate ways to measure these values take advantage of simpler techniques such as reverse-phase liquid chromatography, wherein retention times can be correlated to partition coefficient (Veith, G.D., et al., *Water Research* 13:43-47 1979), as described in Example 1 below.

One measures the partition coefficient and solubility characteristics of the invention oligonucleotide analogs by procedures known in the art and exemplified below. The presence of these properties provides characterization of oligomers that are capable of efficient passive diffusion across cell membranes.

Utility and Administration

Since the oligonucleotides of the invention are capable of passive diffusion across cell membranes they can be used to visualize and label cells and intracellular organelles or other structures. For this use, the oligonucleotides of the invention are provided with a detectable label, such as a radiolabel, fluorescent label, chromogenic label, or enzyme label, and are contacted with cells to be visualized. After a suitable incubation period of about 15 minutes to 2 hours, usually at about 25 to 35°C, the solution containing the labeled oligonucleotides is removed and the cells are washed to remove any unincorporated oligonucleotide. The cells are then prepared for visualization by fluorescence microscopy and detected by visualization of the labeled oligonucleotide. For example, for a fluorescent labeled oligonucleotide, the cells can be plated on a microscope slide and visualized directly.

In addition to employing the oligonucleotides of the invention to visualize cells, the oligonucleotides of the invention are useful in therapy and diagnosis.

Those oligonucleotides that are capable of significant single-stranded or double-stranded target nucleic acid binding activity to form duplexes, triplexes or other forms of stable association, or which bind specific target substances, such as proteins, are useful in diagnosis and therapy of conditions that are

associated with these targets. For example, one or more genes associated with viral infections due to say, HIV, HCMV, HSV or HPV may be targeted. Other therapeutic applications may employ the oligomers to specifically inhibit the expression of genes that are associated with the establishment or maintenance of a pathological condition, such as those for adhesion molecules, receptor molecules or oncogenes that may be associated with inflammatory conditions, immune reactions or cancer respectively. Diagnostic applications for the oligomers include their use as probes for detection of specific sequences by any standard method.

In therapeutic applications, the oligomers are utilized in a manner appropriate for treatment of, for example, viral infections or malignant conditions. For such therapy, the oligomers can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. The oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binders, wetting agents, disintegrants, surface-active agents, or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for injections, including liposome preparations.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Dosages that may be used for systemic administration preferably range from about 0.01 mg/Kg to 50 mg/Kg administered once or twice per day. However, different dosing schedules may be utilized depending on (i) the potency of an individual oligomer at inhibiting the activity of its target gene, (ii) the severity or extent of a pathological disease state associated with a given target gene, or (iii) the pharmacokinetic behavior of a given oligomer.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be

permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, or suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target substances to which they specifically bind. Such diagnostic tests are conducted by complexation with the target which complex is then detected by conventional means. For example, the oligomers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support detected. Alternatively, the presence of complexes may be detected by antibodies which specifically recognize them. Means for conducting assays using such oligomers as probes are generally known.

The invention oligonucleotides may be used to mark or "tag" various items such as plastics, chemicals (e.g., fertilizers, explosives, gunpowders and fuels), oils and emulsions (e.g., crude or refined oils, mineral oils and cosmetics), and fibers (e.g., synthetic or natural clothing fabrics). In these applications, one optionally mixes the invention oligonucleotides with the item or substance one wants to tag or one covalently links the oligonucleotide to the tagged item or substance. One can detect the presence invention oligonucleotides in various products using known direct detection means such as by detecting the presence of radioactive atoms in the oligonucleotides that are present in unique proportions that are easily identified (e.g., 10 atoms of ^{14}C per 1 atom of ^{3}H). One can detect the invention oligonucleotides using indirect means, e.g., techniques for amplifying small amounts of nucleic acids, i.e. by polymerase chain reaction (PCR) techniques. PCR techniques are known for amplifying nucleic acid analogs, or their derivatives, that one normally can not amplify by standard techniques (see, e.g., PCT US93/07130). When one elects to use PCR to detect invention oligonucleotides using PCR, one will generally use base analogs that retain at least some of their base pairing capacity, i.e., base pairing capacity sufficient to facilitate performing the PCR procedure.

In addition to the foregoing uses, the ability some of the oligomers to inhibit gene expression can be verified in *in vitro* systems by measuring the levels of expression in recombinant systems using described methods (see, e.g., Lewis et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 93:3176-3181 1996; Wagner et al., *Science* 260:1510-1513 1993).

The following examples are intended to further illustrate but not to limit the invention. Applicants incorporate all citations herein with specificity.

EXAMPLE 1

Evaluation of Distribution Coefficient. When a compound is allowed to partition between octanol and water, the concentration of the compound in the octanol divided by the concentration of the compound in the water is commonly referred to as the octanol/water partition coefficient (Poct). This number and the logarithm of the partition coefficient (Log Poct) are useful parameters when describing the permeability of a compound towards its membrane. A modification of the procedure of Veith, G.D., Austin, N.M., and Morris, R.T., *Water Research* 13:43-47 1979 using the HPLC retention time of compounds was used to determine the log of the partition coefficients of oligonucleotides (Log Poct). Essentially, the partition coefficients for compounds with unknown Log Poct may be determined by comparison of retention times of the desired compounds with compounds of known Log Poct. The HPLC retention times of a set of standard compounds having known Log Poct values was used to generate a plot of Log Poct versus Log k', where k'=[tr-to]/to. (Here tr = retention time, and to = void time). The resulting plot was fit to a third degree polynomial curve using Cricket Graph software. A typical equation for the curve was y=-32.376 + 102.51x - 107.01x2+37.810x3. Typical R^2 values were R^2=0.999.

The column used was a Hamilton PRP-1, 10 micron, 150 x 4.6 mm ID column. Solvent buffers used were: Solution A: 5 mM potassium phosphate in 2% CH_3CN in H_2O, pH=7.4, and Solution B: 85% CH_3CN in H_2O. A flow rate of 1 mL/min was used. A linear gradient was used that went from 0% to 100% solution B in 30 min. Detection was monitored at 254 and 500 nanometers. Stock solutions of five standards were made up as 100 OD (A260 units)/mL solutions in 50% aqueous CH_3CN. Then 10 OD (A260)/mL solutions were made from the stock solutions by dilution with H_2O. Void

times (to) were calculated by injecting MeOH and monitoring for the first baseline disturbance. These values were typically k'=1.45 min.

The five standard compounds used for the determination of the curve were 3-aminophenol (Log Poct=0.17), 2-aminophenol (Log Poct=0.62), aniline (Log Poct=0.9), o-nitroaniline (Log Poct=1.44), and benzophenone (Log Poct=3.18). The Log Poct values for these compounds were discussed in the paper of Veith et al. above. The five samples were mixed in a 1:1:1:1:3 proportion. Aliquots of 20-50 microliters of this mixture were injected. Their resulting retention times and known Log Poct values were used to generate a curve as described above. Samples with an unknown Log Poct were made up as solutions of 5 OD (A260)/mL in MeOH. Aliquots of 20-40 microliters were injected. A typical standard curve is shown for the five standard reference compounds in Figure 1. The retention time was used to calculate a k' value. This k' value and the standard curve were then used to determine the Log Poct value for unknown compounds.

EXAMPLE 2

Determination of Solubility of Oligonucleotide Analogs.

Oligonucleotides were resuspended in water at a stock concentration of 10 μM to 10 mM. The solution was then diluted in the aqueous media such as DMEM tissue culture medium at decreasing concentrations. The microscope was then used to analyze the solution for fine particles, micelles, etc. Solubility was detected at a minimum oligomer concentration of 50 nM. This lower solubility limit was determined by the sensitivity of the fluorescent microscope. This value can be extended to a 10 nM concentration using more sensitive apparatus.

EXAMPLE 3

Cell staining protocol. Oligonucleotides with various base or backbone modifications were synthesized with one of a variety of amino-linkers. These linkers included "5'-amino-modifier C6" (Glen Research; cat. no. 10-1906), "amino-modifier dT" (Glen Research; cat. no. 10-1039), and "3'-amino-modifier CPG" (Glen Research; cat. no. 20-2950). The following fluors were bonded to the oligomers to monitor uptake: tetramethylrhodamine, resorufin, fluorescein, BODIPY (Molecular Probes) and acridine. A number of other fluors including dansyl, various coumarins, bimane, and pyrene have been evaluated as potential fluorescent probes, however these did not have a bright enough signal (relative quantum yield) to

enable further investigation. A preferred fluor is fluorescein. This dye is itself permeant to most of the cell types tested, giving a total cellular fluorescence. Within 15 min after washing the dye away from the exterior of the cells, the intracellular pool of the dye is pumped out, either by an organic anion pump mechanism or by diffusion. Fluorescein was conjugated to all of the linkers (without oligonucleotide) used and these conjugates were shown to retain the same biological properties. This fluor is very fluorescent, it does however quench rapidly. It is also pH sensitive, being greater than an order of magnitude less fluorescent at pH 5.0 than at pH 7.5. At pH 5.0 the molecule has a net neutral charge, at pH 7.5 it has a net negative charge. BODIPY, which has desirable molecular characteristics such as a neutral charge at cellular pH ranges, lower molecular weight than fluorescein and a greater quantum yield than fluorescein is also a preferred fluor.

Fluorescent measurements were made using a Zeiss Axiovert 10 microscope equipped with a 50W mercury arc lamp and outfitted with a set of fluorescent filters available from Omega Optical (Burlingtion, VT, USA). Observations were made from live cells with a 63x or 100x objective (culture chamber and conditions described below). Photographs were taken with Tri-X / ASA 400 Kodak film and developed with Diafine developer (ASA rating 1600). Exposure time was fixed at 15 to 60s to enable direct comparison.

Fluorescent measurements were also made using a Nikon Diaphot inverted microscope equipped with a phase 4 long working distance condensor, 100W mercury arc lamp, Omega optical fluorescent filters, 40x, 60x and 100x PlanApochromat phase/oil-immersion objectives, and 100% transmission to the video port. A Quantex high-intensity/intensified CCD camera was used to digitize the fluorescent information. This information was sent to a Data Translations FrameGrabber board mounted on a Macintosh II CPU. The Macintosh II was equipped with 8MB RAM and had attached to it a 330MB hard drive. Images were recorded using public domain NIH software "IMAGE". Linearity of information was established using a series of neutral density filters. Relative fluorescent intensity was compared between samples using the same camera settings and variable neutral density filters.

Optimal fluorescence measurements were made using a confocal microscope imaging system which optically slices "sections" through a cell. A Noran real-time confocal imaging optical path equipped with a 3-line (457nm, 488nm, 529nm) laser which is hooked up to the Zeiss Axiovert 10 inverted microscope described above was used. The imaging system was the Macintosh II system described above.

The cell staining assay utilized various cell lines and included P388D1 (mouse macrophage), HEPG2 (human liver), CV1 (monkey epithelial), ccd50sk (untransformed human fibroblast), Rat2 (rat fibroblast), MDCK (kidney cells), L6 (rat myoblast), L cells (mouse fibroblast), HeLa (human adenocarcinoma), skov3 (human ovarian adenocarcinoma), and skbr3 (human breast adenocarcinoma) cells. Other cell lines that were used included Jurkat (human T cell), H9 (human T cell), NIH3T3 (mouse fibroblast), HL60 (human T cell), and H4 (rat liver). All cell lines are commercially available from the American Type Culture Collection, Rockville, MD.

Cells were grown on 25mm-#1 coverslips in media containing 25mM HEPES, pH 7.3, (which helps maintain pH on the microscope) without phenol red (which can lead to high background fluorescence when working with living cells). Coverslips were used so that the high numerical aperture oil-immersion lenses on the microscope could be used. The coverslips were mounted onto "viewing chambers": 6-well petri dishes which have 22mm holes drilled into the bottom. The slides were mounted with silicon vacuum grease which was shown to be non-toxic to the cells. 12x12 mm glass rasching rings (Stanford Glassblowing Laboratory, Stanford, CA) were mounted directly onto the coverslip using paraffin wax. The chamber permitted the use of incubation volumes less than 200 µL. Fluorescent oligonucleotide conjugates were added at concentrations ranging from 0.1 to 150 µM. Stock concentrations of oligonucleotides were prepared in 25mM HEPES, pH 7.3. Oligonucleotides were added to media with or without 10% 4hr-heat inactivated (567C) fetal bovine serum.

Incubation times ranged from 15 minutes to 24 hours. 2 hour incubations were generally utilized for cell staining. Cells were then extensively washed to remove extracellular oligomer using media and observed at room temperature. Slides were optionally replaced in the incubator and were observed over the following 48-72 hours.

EXAMPLE 4

Subcellular Compartment Staining. Fluorescent oligomer compounds were placed on fibroblasts, hepatocytes, muscle and carcinoma cell lines at 50 µM for 2 hours at 37°C; the cells were washed with cell media and live cells were visualized for cellular staining using fluorescent confocal microscopy. The results obtained for representative compounds were:

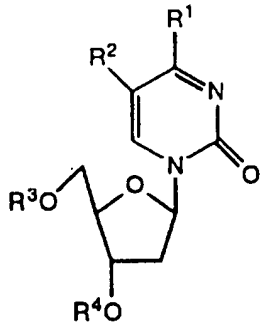
Compound	Log Poct*	Cellular Compartment Stained
223-19C	ND	Mitochondria
183-53	0.26	Cytoplasmic/nucleus
223-4D	1.61	Endoplasmic reticulum/nuclear envelope
156-71A	2.09	Cytoplasmic/nucleus
156-31F	ND	Outer membrane
223-98E	1.14	Cytoplasmic/nucleus
273-21D	1.86	Cytoplasmic/nucleus
273-22D	2.18	Cytoplasmic/nuclear stain

* Log Poct at pH 7.4; ND, not determined

The structures of the listed compounds are given in Figure 2. All of the listed compounds were soluble in aqueous solution to the extent that they could be visualized by fluorescence microscopy. Each compound entered cellular cytoplasm rapidly after addition to cells in tissue culture. As indicated in Figure 2, the molecular weight of the compounds ranged from 846 daltons to 3484 daltons and, in the case of compound 273-22D, carried a negative charge. These results are the first examples known by the present inventors of efficient passive diffusion by oligonucleotide analogs into cells.

EXAMPLE 5

Synthesis of Monomers. The following compounds of the formula



are shown in Table 1 and synthesized as described below.

Table 1

Compound	R^1	R^2	R^3	R^4
1	OH	$-C\equiv C-CH_2CH_2CH_3$	H	H
2	OH	$-C\equiv C-CH_2CH_2CH_3$	DMT	H
3	OH	$-C\equiv C-CH_2CH_2CH_3$	DMT	HPO_2^- $HTEA^+$
4	OH	$-CH_2CH_2CH_2CH_2CH_3$	H	H
5	OH	$-CH_2CH_2CH_2CH_2CH_3$	DMT	H
6	OH	$-CH_2CH_2CH_2CH_2CH_3$	DMT	HPO_2^- $HTEA^+$
7	OH	$-CH_2CH_2CH_2CH_2CH_3$	DMT	$-CH_2S-CH_3$
8	OH	$-CH_2CH_2CH_2CH_2CH_3$	H	TBS
9	$OCH_2CH_2CH_2CH_3$	$-CH_3$	DMT	H
10	$OCH_2CH_2CH_2CH_3$	$-CH_3$	DMT	HPO_2^- $HTEA^+$

DMT = 4,4'-dimethoxytrityl
TBS = t-butyldimethylsilyl
HTEA+ = hydrogentriethylammonium

5-(1-Pentynyl)-2'-deoxyuridine (1). This compound was prepared by the same procedure that Hobbs, F.W.J., *J. Org. Chem.* 54:3420-3422 1989, used for the preparation of other alkynyl substituted nucleosides. A mixture of 30.0 †g (84.7 mmol) of 5-iodo-2'deoxyuridine (purchased from Sigma), 23.6 mL of 1-pentyne (Aldrich), 9.79 g of tetrakis (triphenylphosphine) palladium (0) (Aldrich), and 3.23 g of copper (I) iodide were stirred at room temperature for 26 h. To the reaction was added 250 mL of MeOH and 250 mL of CH_2Cl_2. The mixture was neutralized with Dowex 1 x 8-200 (bicarbonate form) ion exchange resin. The mixture was filtered and concentrated. The residue was partitioned between H_2O and CH_2Cl_2. The aqueous layer was extracted three times with CH_2Cl_2 and then concentrated. Purification of the crude product by column chromatography afforded 20.4 g (81.9% yield) of product.

5'-O-(4,4'-Dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridine (2). To 20.4 g (69.3 mmol) of 5-(1-pentynyl)-2'-deoxyuridine in 300 mL of dry pyridine was added 22.8 g of 4,4'-dimethoxytrityl chloride. The reaction was stirred for 17 h at room temperature and then concentrated. The residue was taken up in CH_2Cl_2 and washed twice with 0.5% aqueous $NaHCO_3$, dried (Na_2SO_4),

filtered, and concentrated. Purification of the crude product by column chromatography afforded 21.9 g (52.9% yield) of product.

5'-O-(4,4'-Dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt (3). To an ice-cold solution of 1.36 g (2.28 mmol) of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridine in 7.39 mL of dry pyridine and 17.8 mL of dry CH_2Cl_2 was added 9.30 mL of a 1.00 M solution of 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (purchased from Aldrich as a solid) in CH_2Cl_2, dropwise over two minutes. The reaction was stirred at 0°C for 45 minutes and then poured onto a rapidly-stirred, ice-cooled mixture of 62 mL of 1 M aqueous triethylammonium bicarbonate (TEAB, pH = 8.2) and 31 mL of CH_2Cl_2. The mixture was stirred for 15 minutes and the layers were separated. The organic layer was washed with 1 M aqueous TEAB, dried (Na_2SO_4), filtered, and concentrated. After isolation of the product by column chromatography on silica gel, the product was taken up in CH_2Cl_2, washed with 1 M aqueous TEAB, dried (Na_2SO_4), filtered, and concentrated. This procedure afforded 938 mg (53.9% yield) of product.

5-Pentyl-2'-deoxyuridine (4). To a solution of 1.03 g (3.50 mmol) of 5-(1-pentynyl)-2'-deoxyuridine in 25 mL of MeOH was added a catalytic amount of 10% Pd on charcoal. The mixture was hydrogenated under 300 psi of H_2 for 14 hours at room temperature. The mixture was filtered through Celite and concentrated, affording a quantitative yield of product.

5'-O-(4,4'-Dimethoxytrityl)-5-pentyl-2'-deoxyuridine (5). This compound was prepared from 1.04 g (3.49 mmol) of 5-pentyl-2'-deoxyuridine by the same procedure used for the preparation of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridine. Column chromatography of the crude residue on silica gel afforded 1.70 g (81.0% yield) of product.

5'-O-(4,4'-Dimethoxytrityl)-5-pentyl-2'-deoxyuridin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt (6). This compound was prepared from 1.48 g (2.46 mmol) of 5'-O-(4,4'-dimethoxytrityl)-5-pentyl-2'-deoxyuridine by the same procedure used for the preparation of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridine-3'-yl-hydrogenphosphonate hydrogentriethylammonium salt. This procedure afforded 1.35 g (71.8% yield) of product.

5'-O-(4,4'-Dimethoxytrityl)-3'-O-methylthiomethyl-5-pentyl-2'-deoxyuridine (7). To a solution of 3.50 g (5.83 mmol) of 5'-O-(4,4'-dimethoxytrityl)-5-pentyl-2'-deoxyuridine in 148 mL of dry THF was carefully (hydrogen evolution!) added 835 mg of sodium hydride (97%) in small portions at room temperature. After stirring the mixture for 30 minutes, 959 mg of sodium iodide (NaI) was added, followed by 0.557 mL of chloromethyl methyl sulfide (Aldrich). The reaction was stirred for 4 h and then carefully quenched with MeOH. The mixture was concentrated. The residue was partitioned between CH_2Cl_2 and H_2O, shaken, and separated. The organic layer was washed with sat. aqueous $NaHCO_3$, H_2O, dried (Na_2SO_4), filtered, and concentrated. The crude residue was purified by column chromatography on silica gel affording 2.76 g (71.7% yield) of product.

3'-O-t-Butyldimethylsilyl-5-pentyl-2'-deoxyuridine (8). To a mixture of 3.50 g (5.83 mmol) of 5'-O-(4,4'-dimethoxytrityl)-5-pentyl-2'-deoxyuridine and 1.91 g of imidazole in 23.3 mL of dry DMF was added 1.05 g of t-butyldimethylsilyl chloride (purchased from Petrarch). The reaction was stirred at room temperature for 20 h and then concentrated. The residue was partitioned between CH_2Cl_2 and H_2O, shaken, and separated. The organic layer was washed with H_2O, and concentrated. The crude material was stirred in 150 mL of 80% HOAc in H_2O for 3 h and then concentrated. The residue was taken up in CH_2Cl_2 washed with H_2O, saturated aqueous $NaHCO_3$, dried (Na_2SO_4), filtered, and concentrated. Column chromatography of the crude residue afforded 1.88 g (78.3% yield) of product.

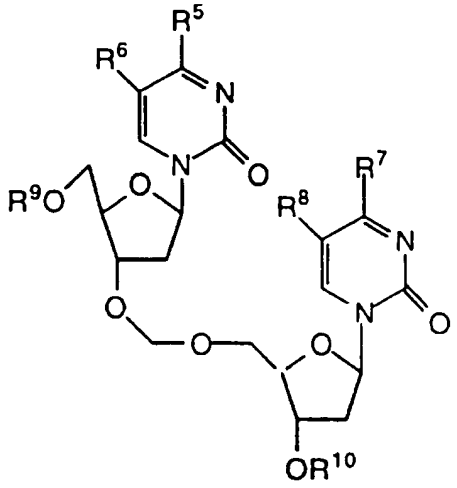
4-O-Butyl-5'-O-(4,4'-dimethoxytrityl)-thymidine (9). To an ice-cold solution of 5'-O-(4,4'-dimethoxytrityl)-thymidine (2.0 g; 3.67 mmole) in 20 mL of CH_2Cl_2 was added 6 mL of N,N-dimethylaminotrimethylsilane. After stirring 30 min. at 0°C, the reaction mixture was concentrated to dryness. The crude residue was dissolved in 50 mL of acetonitrile. To this was added triethylamine (11 g; 110 mmoles) and 1,2,4-triazole (1.52 g; 22 mmoles), and the mixture cooled to 0°C. To this ice-cold mixture was added $POCl_3$ (1.10 g; 7.3 mmole). The reaction mixture was stirred at 0°C for 3 h, then at room temperature overnight. The reaction was then concentrated. The residue was dissolved in CH_2Cl_2, and washed twice with saturated aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography on silica gel, affording 2.20 g of triazole intermediate. The triazole intermediate (2.1 g; 3.5 mmole) was dissolved in

anhydrous n-butanol (12 mL) and treated with DBU (1.0 g; 7.0 mmole). After one h, the reaction mixture was concentrated to dryness. The residue was dissolved in CH_2Cl_2, washed with 10% aqueous citric acid, dried over Na_2SO_4, and filtered. The residue was purified by column chromatography on silica gel, affording 1.0 g of product.

4-O-Butyl-5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt (10). This compound was prepared from 4-O-butyl-5'-O- (4,4'-dimethoxytrityl)-thymidine in the same manner as described for the preparation of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt.

EXAMPLE 6

Synthesis of Dimer Synthons Containing Formacetal Linkages. The following dimers of the formula



are shown in Table 2 and synthesized as described below.

Table 2

Compound	R^5	R^6	R^7
11	OH	$-CH_2CH_2CH_2CH_2CH_3$	OH
12	OH	$-CH_2CH_2CH_2CH_2CH_3$	OH
13	OH	$-CH_3$	OH
14	$OCH_2CH_2CH_2CH_3$	$-CH_3$	$OCH_2CH_2CH_2CH_3$
15	$OCH_2CH_2CH_2CH_3$	$-CH_3$	$OCH_2CH_2CH_2CH_3$

Compound	R^8	R^9	R^{10}
11	$-CH_2CH_2CH_2CH_2CH_3$	DMT	H
12	$-CH_2CH_2CH_2CH_2CH_3$	DMT	HPO_2-HTEA$^+$
13	CH_3	DMT	H
14	CH_3	DMT	H
15	CH_3	DMT	HPO_2-HTEA$^+$

For abbreviations, see Table 1

5'-O-([5'-O-(4,4'-Dimethoxytrityl)-5-pentyl-2'-deoxyuridin-3'-O-yl]-methyl-5-pentyl-2'-deoxyuridine (11). This compound was prepared from compounds 7 and 8 in the same manner as that previously described for the preparation of 5'-O-([5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-thymidine in U.S. Patent No. 5,264,562 in 86% yield.

5'-O-([5'-O-(4,4'-Dimethoxytrityl)-5-pentyl-2'-deoxyuridin-3'-O-yl]-methyl)-5-pentyl-2'-deoxyuridin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt (12). This compound was prepared from 5'-O-([5'-O-(4,4'-dimethoxytrityl)-5-pentyl-2'-deoxyuridin-3'-O-yl]-methyl-5-pentyl-2'-deoxyuridine using the same procedure described for the preparation of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt.

5'-O-([5'-O-(4,4'-Dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-thymidine (13). This compound was prepared as described in U.S. Patent No. 5,264,562.

5'-O-([4-O-Butyl-5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-4-O-butylthymidine (14). This compound was prepared from 5'-O-([5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-thymidine by the same procedure used for the preparation of 4-O-butyl-5'-O-(4,4'-dimethoxytrityl)-thymidine. Column chromatography afforded a 52% yield of product.

5'-O-([4-O-Butyl-5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-4-O-butylthymidin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt (15). This compound was prepared from 5'-O-([4-O-butyl-5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl-4-O-butylthymidine by the same procedure used for the preparation of 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl-2'-deoxyurdin-3'-O-yl-hydrogenphosphonate hydrogentriethylammonium salt.

EXAMPLE 7

Synthesis of Oligonucleotides - General Procedures. The pivaloyl chloride (trimethylacetylchloride) was purified by distillation at atmospheric pressure and stored under argon. The solvents (pyridine, dichloromethane, acetonitrile) were dried over activated molecular sieves (3Å). The solvents used in the coupling cycle should be as anhydrous as possible to avoid any undesirable hydrolysis reactions. The starting dimethoxytrityl protected deoxynucleoside H-phosphonates were dried by co-evaporation from anhydrous acetonitrile and subsequently reconstituted in 1:1 anhydrous pyridine and acetonitrile. Synthesis was performed with the aid of a Biosearch Model 8700 DNA synthesizer employing solid support, preferably CPG (controlled pore glass).

Functionalization of Solid Support. To a solution of an appropriate nucleoside (such as 5'-O-(4,4'-dimethoxytrityl)-5-(1-pentynyl)-2'-deoxyuridine, 5'-O-(4,4'-dimethoxytrityl)-5-pentyl-2'-deoxyuridine, 4-O-butyl-5'-O-(4,4'-dimethoxytrityl)- thymidine, 5'-O-([5'-O-(4,4'-dimethoxytrityl) -5-pentyl-2'-deoxyuridin-3'-O-yl]-methyl)-5-pentyl-2'-deoxyuridine, 5'-O-([4-O-Butyl-5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-4-O-butylthymidine, or 5'-O-([5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-O-yl]-methyl)-thymidine) in 12 mL of anhydrous pyridine containing triethylamine (TEA, 80 µL) was added 384 mg of DEC [1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride], 12 mg of DMAP (N,N-dimethylaminopyridine), and 1 g of CPG LCAA succinic acid (LCAA, long chain alkyl amine). The resulting mixture was sealed under argon, wrapped in foil, and shaken for 14

hours. The amount of nucleoside loading was determined by the dimethoxytrityl cation assay described below.

Dimethoxytrityl Cation Assay for the Determination of Nucleoside Loading on Solid Support. To 1 mg of functionalized CPG was added 1 mL of 0.1 M p-toluenesulfonic acid monohydrate (TSA) in dichloromethane. The UV absorption of the solution using a standard cell was then measured at 498 nm. The degree of substitution (loading) was calculated using the following formula: substitution (μmole/g) = A498 x 14.3, where A = absorbance.

Nucleoside substitutions (loadings) achieved were typically between 20 and 40 μmole of nucleoside per gram of functionalized support. The unreacted succinic acid sites on the solid support were capped by adding 134 mg of pentachlorophenol and shaking the mixture for 16 h. This formed the corresponding ester. The mixture was filtered and the support was sequentially washed with pyridine, dichloromethane, and then diethylether. The support was then shaken with 10 mL of anhydrous piperidine in a 25 mL round bottomed flask for 5 min. The mixture was filtered and the support washed with dichloromethane and then diethylether. The support was then added to an anhydrous solution containing 2.5 mL of acetic anhydride, 10.0 mL of pyridine, and 10 mg of DMAP. The solution was placed under argon, capped, and shaken for 4 h. The mixture was filtered, and the functionalized CPG was washed sequentially with pyridine, dichloromethane, methanol and diethylether. The CPG was dried in vacuum and was then ready for solid phase oligonucleotide synthesis.

Preparation of DNA H-phosphonate. The oligonucleotide H-phosphonate having the following structures were prepared according to the following procedure. First, the functionalized (A for sequence A, B for sequence B) solid support was placed in a reactor vessel (column) and was washed with dichloromethane. Then, a 2.5% solution of dichloroacetic acid (DCA) in dichloromethane was introduced to remove the 5' protecting group of the support-bound nucleoside. After the deprotection step, the solid support was washed with dichloromethane, and then anhydrous pyridine/acetonitrile (1/1, by volume).

The first coupling cycle was initiated by the addition of a 1.5% solution of pivaloyl chloride in anhydrous pyridine/acetonitrile, 1/1) and ten equivalents (based on the amount of loading of the support bound nucleotide) of the appropriate protected nucleoside hydrogenphosphonate in anhydrous pyridine/acetonitrile (1/1) in alternating pulses. The reagents were allowed to react for 3.5 min.

At this point the oligonucleotide could be further extended by repeating the sequence of DCA deprotection and pivaloyl chloride coupling until the desired length and sequence of bases was attained. Alternatively, the linkage or linkages could be oxidized to the thiophosphate, phosphodiester or the phosphoramidate.

The final coupling for fluorescent labelling utilizes coupling of 6-N-(4-methoxytrityl)-aminohexan-1-O-yl)-hydrogenphosphonate hydrogen-triethylammonium salt. The coupling of this hydrogenphosphonate was identical to the other hydrogenphosphonate couplings. After coupling and desired oxidation, the monomethoxytrityl protecting group was removed from the amine in a similar fashion as described above.

Conjugation of 5'Amino Linker Oligonucleotide with a Fluorescein Label. A 10 μmole reaction (calculated from the loading of the CPG in μm/g and the mass of the support bound nucleoside) was placed in 3.6 mL of anhydrous N,N-dimethylforamide (DMF) and 0.4 mL of disopropylethylamine. To this solution was added 24 mg of 5- (and 6-) carboxyfluorescein, succinimidyl ester. The reaction was capped and shaken in the dark for 10 h, and then filtered. The solid support was then sequentially washed with dichloromethane, DMF, water, methanol and then diethylether. The support-bound, fluorescently-labelled oligonucleotide was then washed to remove unconjugated carboxyfluorescein.

Oxidation of the Oligonucleotide H-Phosphonate to the Thiophosphate. The DNA H-phosphonate, prepared above, was converted directly to the thiophosphate, preferably while the DNA was still bound to the solid support, by the addition to the reactor vessel of 1 mL of an oxidizing mixture comprised of a 2.5% solution (by weight) of elemental sulfur (sublimed sulfur powder available from Aldrich Chemical Company, Milwaukee, Wisconsin, USA, Cat No. 21,523-6) in anhydrous pyridine/carbon disulfide (1/1, v/v). The contents of the reactor were mixed for 20 min., and then the reagents were removed. This oxidation cycle was carried out a second time using 1 mL of an oxidizing solution comprising equal volumes of a 2.5 wt% solution of elemental sulfur in anhydrous pyridine/carbon disulfide (1/1, v/v) and 10% by volume diisopropylethylamine in anhydrous pyridine. Finally, the oxidized copolymer-bound oligonucleotide was washed with anhydrous pyridine/acetonitrile (1/1, v/v), followed by anhydrous dichloromethane.

Oxidation of the Oligonucleotide H-Phosphonate to the Phosphodiester and the Phosphoramidate Analog. The oligonucleotide H-phosphonate was

oxidized, when desired, to the phosphodiester derivative by the following procedure:

Method A: To the solid support, obtained from the process outlined above, was added 1 mL of an oxidizing solvent mixture comprised of 0.1 M I_2 in water/pyridine (2/98, v/v). The resulting mixture was agitated for 15 min., and then the reagents were removed. Afterwards, 1 mL of a second oxidizing solvent mixture made from equal volumes of 0.1 M I_2 in water/pyridine (2/98, v/v) and 0.1 M triethyl ammonium bicarbonate in water/pyridine (1/9, v/v) was added to the solid support. After mixing the contents of the reactor for 5 min., the reagents were removed. Finally, the oxidized copolymer-bound product was washed with anhydrous pyridine/acetonitrile (1/1, v/v) and then anhydrous dichloromethane.

Method B: Alternatively, the oligonucleotide H-phosphonate was oxidized to the phosphoramidate analog by the following procedure: To the solid support, obtained from the procedure outlined above, was added 18 mL of an oxidizing solvent mixture made from 10% by volume of the desired amine in anhydrous/pyridine/carbon tetrachloride (1/1, v/v). The resulting mixture was agitated for 15 min., after which time the spent oxidizing solvent mixture was discarded. Finally, the oxidized copolymer-bound product was washed with anhydrous pyridine/acetonitrile (1/1, by volume), and then anhydrous dichloromethane.

The oligonucleotide H-phosphonates could be oxidized or converted to a number of other linkage derivatives, such as phosphoric acid triesters, dithiophosphoric acids, their corresponding esters and amidates, and other which are desirable to and which are within the skill of those knowledgeable in the art. Related oxidation procedures are described, for example, in application no. EP 0 219 342, the complete disclosure of which is incorporated herein by reference. Thus, oligonucleotides having a variety of linkages derived from phosphoric acid, such as phosphoric acid diesters, phosphoric acid triesters, thiophosphoric acid, dithiophosphoric acid, phosphoric acid thioesters, phosphoric acid dithioesters, phosphoric acid amidates, or thiophosphoric acid amidates, can be readily obtained from the methods described above.

Cleavage of the Oligonucleotide From the Copolymer Support. Once the synthesis of the oligonucleotide was complete, the DNA was cleaved from the solid support, with the concurrent removal of any base protecting groups, by the addition of concentrated aqueous ammonium hydroxide and heating

the resulting mixture at 45°C for 24 h. The product oligonucleotide was washed from the solid support with methanol/water.

Purification was effected by reverse-phase HPLC, under the conditions described further below.

HPLC Purification of the Fluorescently Labeled Oligonucleotide. A crude sample containing approximately 10 Tmole of the fluorescently labeled oligonucleotide, prepared by the methods described above, and dissolved in a solvent mixture of 1/1 (v/v) methanol/water (10 mL) was concentrated under vacuum. The oligonucleotide was resuspended in 1 mL of methanol and then diluted with 100 mM aqueous triethylammonium acetate (TEAA, pH 7.0) and 5% (by volume) aqueous acetonitrile to a final volume of 10 mL. This dilute oligonucleotide solution was then loaded, at a flow rate of 2 mL/min, on a Septech A/E 160 cm x 10 cm i.d., column packed with Hamilton PRP-1 (polystyrene stationary phase, 12-20 μm), which had been preconditioned with 60 mL of a buffer solution comprised of 5% by volume of acetonitrile in 100 mM TEAA (Buffer C, pH=7.0) at a flow rate of 3 mL/min. After the completion of the sample loading a 45 min. linear gradient to 100% of Buffer D (75% by volume acetonitrile in 100 mM TEAA, pH=7.0) was initiated. After 45 min., a linear gradient of 100% Buffer B in 15 min. (100% acetonitrile) was initiated. The product was eluted and collected. The collected fractions were then dried in vacuum, and the excess TEAA salt was removed by co-evaporation 3x with 1 mL 90% ethanol, 10% water. The counter ion (if present) was then exchanged by passing the oligonucleotide (in 0.5 mL) over a Poly-Prep, Bio-Rad column (packing AG 50 w x 8 Na form) and eluting with 3 mL of water to yield a highly pure fluorescently labeled oligonucleotide.

Applicants incorporate all citations herein with specificity. Applicants incorporate all citations herein by reference.

Claims

We claim:

1. An oligonucleotide wherein the oligonucleotide comprises internucleotide linkages, bases and sugars and wherein the oligonucleotide has an octanol:water partition coefficient of -0.3 to +2.5 and a solubility in water of at least 0.001 μg/mL, and the and salts, solvates and hydrates thereof.

2. The oligonucleotide of claim 1 wherein the octanol:water partition coefficient is 0.0-2.5.

3. The oligonucleotide of claim 1 wherein the oligonucleotide contains at least one C_{1-8} hydrocarbyl substituent or C_{1-14} pseudohydrocarbyl substituent, and wherein the oligonucleotide optionally contains 0-4 C_{9-16} hydrocarbyl substituents, or 0-4 C_{14-18} pseudohydrocarbyl substituents bonded to the linkages, bases or sugars.

4. The oligonucleotide of claim 3 wherein the hydrocarbyl or pseudohydrocarbyl substituents are bonded to at least at 60% of the linkages, bases or sugars, or wherein at least 60% of the linkages, bases and sugars are substituted with hydrocarbyl or pseudohydrocarbyl.

5. The oligonucleotide of claim 4 wherein the hydrocarbyl or pseudohydrocarbyl substituents are C_{1-8} hydrocarbyl or C_{1-14} pseudohydrocarbyl substituents.

6. The oligonucleotide of claim 3 wherein the hydrocarbyl or pseudohydrocarbyl substituents are bonded to 60-90%, of the linkages, bases or sugars, or wherein at least 60-90% of the linkages, bases and sugars are substituted with hydrocarbyl or pseudohydrocarbyl.

7. The oligonucleotide of claim 1 wherein
at least 60% of the linkages are lipophilic, or
at least 60% of the bases are lipophilic, or
at least 60% of the sugars are lipophilic, or
at least 60% of the linkages, bases and sugars are lipophilic.

8. The oligonucleotide of claim 7 wherein
at least 80% of the linkages are lipophilic, or
at least 80% of the bases are lipophilic, or
at least 80% of the sugars are lipophilic, or
at least 80% of the linkages, bases and sugars are lipophilic.

9. The oligonucleotide of claim 7 wherein the oligonucleotide contains ionic, non-ionic or a mixture of ionic and non-ionic linkages and
at least 120% of the internucleotide linkages, sugars and bases contain a lipophilic substitution, or
at least 120% of the bases and sugars contain a lipophilic substitution, or
at least 120% of the linkages and the bases contain a lipophilic substitution.

10. The oligonucleotide of claim 1 wherein the linkages are selected from the group consisting of formacetal, 3'-thioformacetal, phosphodiester, phosphorothioate, phosphorodithioate, a phosphoramidate containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent, a phosphotriester containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent, and a thiophosphotriester containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent.

11. The oligonucleotide of claim 4 wherein each hydrocarbyl or pseudohydrocarbyl substituent linked to any base independently is selected from the group consisting of C_{1-8} hydrocarbyl and C_{1-8} pseudohydrocarbyl substituents at C-5 of pyrimidines, N2 or C8 of guanine, N6 or C8 of adenine, C7 of 7-deazaguanine, C7 of 7-deazaadenine, N4 of cytosine or C7 and 7-deazapurines.

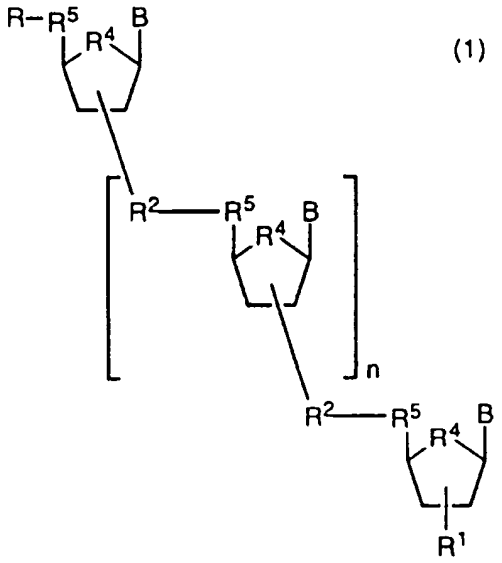
12. The oligonucleotide of claim 1 wherein the oligonucleotide contains at least one nucleoside selected from the group consisting of thymidine, 2'-deoxycytidine, 2'-deoxy-5-methylcytidine, N6-methyl-8-oxo-2'-deoxyadenosine, 2'-deoxy-5-vinylcytidine, 2'-deoxy-5-ethynylcytidine, 2'-deoxy-5-vinyluridine, 2'-deoxy-5-(1-propynyl)cytosine, 2'-deoxy-5-(1-propynyl)uridine, 2'-deoxy-5'-ethynyluridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxy-N7-deaza-7-(1-propynyl)-guanosine and 2'-deoxy-N7-deaza-7-(1-propynyl)-adenosine.

13. The oligonucleotide of claim 1 wherein the oligonucleotide contains 4-15 nucleotide monomers.

14. The oligonucleotide of claim 1 which does not comprise a steriod or a cholesteryl moiety.

15. The oligonucleotide of claim 1 which does not comprise a protecting group.

16. The oligonucleotide of claim 1 having structure (1)



(1)

wherein

R is OH, blocked OH, $N(R^{14})_2$, $P(O)(R^{15})_2$, or a linker;

R^1 is an oligonucleotide, a blocking group, OH, $N(R^{14})_2$, $P(O)(R^{15})_2$, a solid support, or a linker bonded to the 2' or 3' position of a furanose ring or its carbocyclic analog, and the remaining 2' or 3' position is substituted with R^3;

each R^2 independently is an internucleotide linkage bonded to the 2' or 3' position, and the remaining 2' or 3' position is substituted with R^3;

each R^3 independently is H, OH, F, blocked hydroxyl, $N(R^{14})_2$, -O-alkyl (C_{1-8}), -O-alkyl (C_{1-8}) where the alkyl group is substituted with halogen, hydroxyl or oxygen, -O-alkenyl (C_{3-8}), -S-alkyl (C_{1-8}) or a linker;

each R^4 independently is O or CH_2;

each R^5 independently is CH_2, NR^6, O, S, SO, SO_2;

each R^6 independently is H, alkyl (C_{1-6}) or alkyl (C_{1-6}) where the alkyl group is substituted with halogen, hydroxyl or oxygen;

each R^{14} independently is hydrogen, a protecting group, hydrocarbyl, or pseudohydrocarbyl;

each R^{15} independently is hydroxyl (OH), blocked hydroxyl, SH, blocked SH, or $N(R^{14})_2$;

n is an integer from 0 to 48; and

each B independently is a base, wherein the total number of bonded monomers designated by the variable n plus any oligonucleotide at R^1 is 2-50.

17. The oligonucleotide of claim 16 wherein the oligonucleotide is modified such that:

at least 60% of the R^2 groups are not phosphodiester linkages; or

at least 60% of B contain a lipophilic group that is independently chosen for each B; or

at least 60% of the sugars contain a lipophilic group that is independently chosen for each sugar; or

at least 60% of the R^2 groups, bases and sugars are not phosphodiester linkages or are lipophilic, and each R^2, base and sugar contains a linkage or lipophilic group that is independently chosen.

18. The oligonucleotide of claim 16 wherein the lipophilic group at each base independently is a C_{1-8} hydrocarbyl group or a or C_{1-8} pseudohydrocarbyl group wherein the C_{1-8} hydrocarbyl group or C_{1-8} pseudohydrocarbyl group is bonded to a purine or pyrimidine base position selected from the group consisting of a C5 position of pyrimidines, the O4 position of thymine, the N6 position of adenine, the C8 position of adenine, the N2 position of guanine, the C8 position of guanine, the N4 position of cytosine and the C7 position of 7-deazapurines.

19. The oligonucleotide of claim 16 wherein each R^2-R^5 independently is a linkage selected from the group consisting of formacetal, 3'-thioformacetal, phosphodiester, phosphorothioate, phosphorodithioate, a phosphoramidate containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent, a phosphotriester containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent, and a thiophosphotriester containing a C_{1-8} hydrocarbyl or C_{1-8} pseudohydrocarbyl substituent.

20. The oligonucleotide of claim 16 wherein the log Poct value of the octanol:water partition coefficient is 0.0-2.5.

21. The oligonucleotide of claim 1 coupled to a label.

22. The oligonucleotide of claim 21 wherein the label is a radiolabel or a fluorescent moiety.

23. The oligonucleotide of claim 1 which is a dimer, trimer or tetramer.

24. A method comprising:

contacting cells to be visualized or detected with the oligonucleotide of claim 1 under conditions wherein diffusion across the cell membrane can occur so as to internalize said oligonucleotide within the cells;

removing from the cells any oligonucleotide which has not diffused across the membrane and become internalized; and

visualizing or detecting the oligonucleotide which has been internalized in the cells to visualize or detect the cells.

25. The method of claim 24 wherein the oligonucleotide has a solubility of at least about 10 nM in water.

26. The method of claim 24 wherein the cells are viable.

27. The method of claim 26 wherein a subcellular compartment of the mammalian cell is stained.

28. The method of claim 27 wherein a subcellular compartment is endoplasmic reticulum, nuclear envelope, nuclei or mitochondria.

29. A method comprising contacting a subcellular component with the oligonucleotide of claim 1 under conditions wherein binding of the oligonucleotide to the component occurs;

separating unbound oligonucleotide from component-bound oligonucleotide; and

detecting the oligonucleotide bound to the subcellular component.

30. A method comprising contacting a cell with the oligonucleotide of claim 1.

PARTITION COEFFICIENT VS RETENTION
ON PRP-1 (195-42)

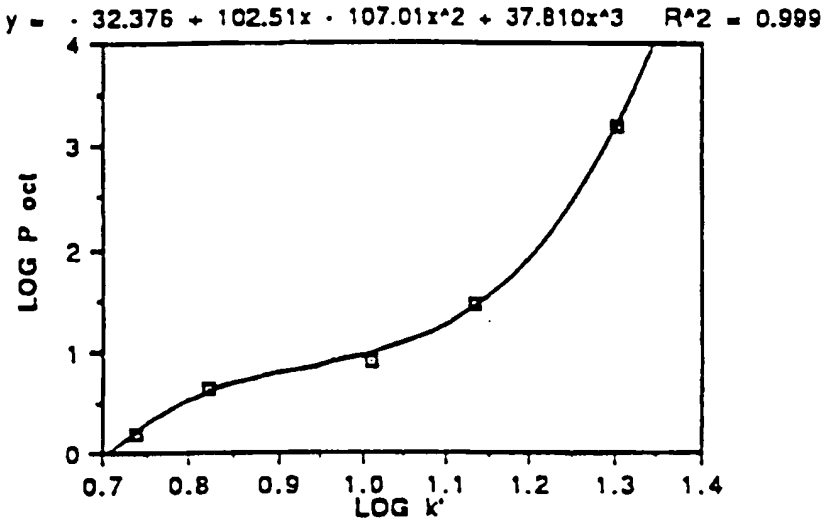


Fig. 1

Compound	Cellular Compartment Stained	Structure*
223-19C 1351 daltons	mitochondria	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_5CH_3$]O- 5' X^2 = 3'-N[$(CH_2)_5CH_3$]-CH_2-CH_2-5' B^1 = B^2 = 4-O-butylthymine
223-4D 1834 daltons	endoplasmic reticulum and nuclear envelope	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_8$-CH_3]O- 5' X^2 = 3'-O-P(O)[NH-$(CH_2)_5CH_3$]O- 5' X^3 = 3'-O-P(O)[NH-$(CH_2)_8CH_3$]O- 5' B^1 = B^2 = B^3 = 5-(1-pentynyl)uracil
156-71A 1520 daltons	cytoplasm and nucleus	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_{11}CH_3$]O- 5' X^2 = 3'-O-P(O)[NH-$(CH_2)_{11}CH_3$]O- 5' B^1 = B^2 = 5-(1-pentynyl)uracil
156-31F 1208 daltons	outer membrane	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_2$NHCO-3-O-cholesterol]O-5' B^1 = thymine

* - linkages (X^1, X^2, etc) are listed starting at the 5' position with X^1 linked to the 5' terminal position; all compounds contain 2'-deoxyribose and contain a free 3' hydroxyl group at the 3' terminal nucleoside residue; bases (B^1, B^2, etc) are listed starting at the 5' terminal nucleoside residue

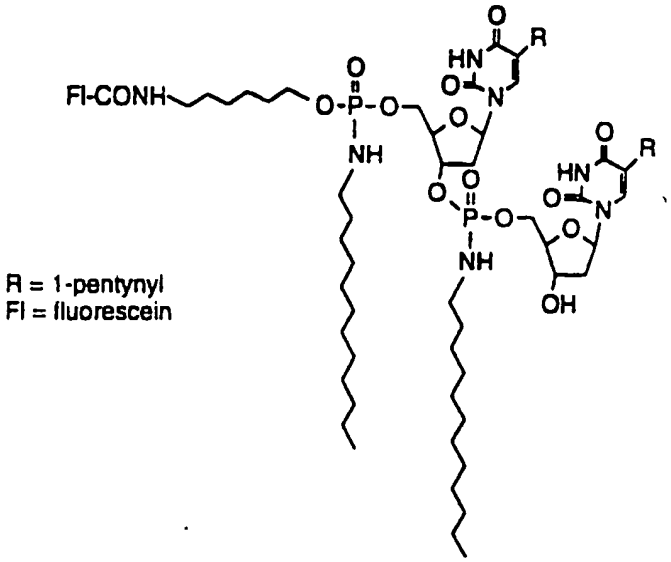
Fig. 2A

Compound	Cellular Compartment Stained	Structure*
183-53 846 daltons	cytoplasm and nucleus	X^1 = fluorescein-C(O)-NH- 5' X^2 = 3'-O-CH_2-O-5' B^1 = B^2 = 4-O-methylthymine
223-98E 2155 daltons	cytoplasm and nucleus	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_5CH_3$]O- 5' X^2 = X^3 = X^4 = 3'-O-P(O)[NH-$(CH_2)_2OCH_3$]O- 5' B^1 = B^2 = B^3 = B^4 = 4-O butylthymine
273-21D 2656 daltons	cytoplasm and nucleus	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_8$-CH_3][NH-$(CH_2)_2OCH_3$]- 5' X^2 = X^4 = X^6 = 3'-O-CH_2-O-5' X^3 = X^5 = 3'-O-P(O)[NH-$(CH_2)_2OCH_3$]O- 5' B^1 = B^2 = B^3 = B^4 = B^5 = B^6 = 4-O-butylthymine
273-22D 3484 daltons	cytoplasm and nucleus	X^1 = fluorescein-C(O)-NH-$(CH_2)_6$-O-P(O)[NH-$(CH_2)_8$-CH_3]O- 5' X^2 = X^4 = X^6 = X^8 = 3' -O-CH_2-O- 5' X^3 = 3' -O-P(O)(O^-)-O- 5' X^5 = 3' -O-P(O)[NH-$(CH_2)_8CH_3$]O- 5' X^7 = 3' -O-P(O)[NH-$(CH_2)_2OCH_3$]O- 5' B^1 = B^2 = B^3 = B^4 = B^5 = B^6 = B^7 = B^8 = 4-O-butylthymine

* - linkages (X^1, X^2, etc) are listed starting at the 5' position with X^1 linked to the 5' terminal position; all compounds contain 2'-deoxyribose and contain a free 3' hydroxyl group at the 3' terminal nucleoside residue; bases (B^1, B^2, etc) are listed starting at the 5' terminal nucleoside residue

Fig. 2B

156-71A



273-22D

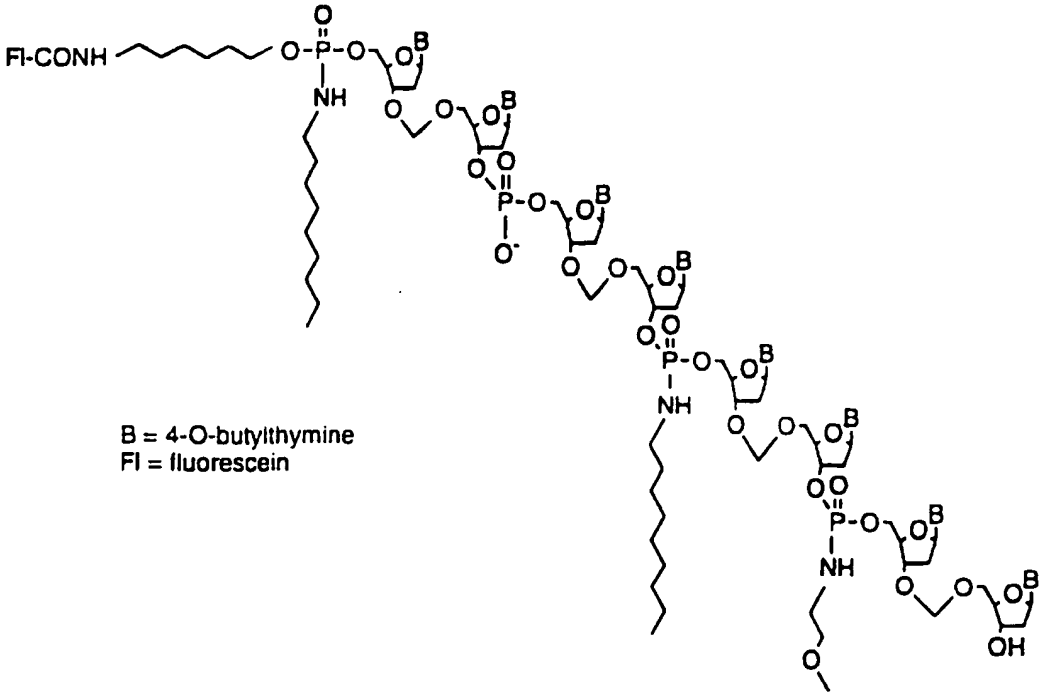


Fig. 2C

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(57) Abstract

The invention discloses lipophilic oligonucleotide analogs that are capable of efficient passive diffusion across cell membranes. These oligonucleotides contain at least two nucleotide residues and have an octanol:water partition coefficient of about -0.3 to + 2.5 and a solubility in water of at least 0.001 μg/mL. Invention embodiments which include lipophilic oligonucleotide analogs having either at least 60 % of the internucleotide linkages are lipophilic, or at least 60 % of the bases contain lipophilic substitutions, or at least 60 % of the sugars contain lipophilic substitutions, or a combination of these sums to 60 %. These oligonucleotides may be conjugated to a label and used to visualize cells or subcellular compartments.

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INTERNATIONAL SEARCH REPORT

Inter inal Application No
PCT/US 96/12530

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 A61K48/00 C12Q1/68 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate of the relevant passages	Relevant to claim No
X	J.F.HUGHES ET AL.: "Lipid Membrane Permeability of 2'-Modified Derivatives of Phosphorothioate Oligonucleotides." JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 83, no. 4, April 1994, pages 597-600, XP002052615 see the whole document ---	1-30
X	US 4 904 582 A (TULLIS RICHARD H) 27 February 1990 cited in the application see column 2, line 25 - column 6, line 48; example 5 ---	1-3,16, 24-30
X	US 5 420 330 A (C.K.BRUSH) 30 May 1995 cited in the application see the whole document ---	1-3,16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 January 1998	03. 02. 98

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter inal Application No
PCT/US 96/12530

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate of the relevant passages	Relevant to claim No
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X	see the whole document	16
A	WO 93 10820 A (GILEAD SCIENCES, INC.) 10 June 1993	1
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A	WO 90 10448 A (GENENTECH INC) 20 September 1990	1
X	see claim 14	16
A	J.M.DAGLE ET AL.: "Physical Properties of Oligonucleotides Containing Phosphoramidate-Modified Internucleoside Linkages." NUCLEIC ACIDS RESEARCH., vol. 19, no. 8, 1991, OXFORD GB, pages 1805-1810, XP002052616 cited in the application see the whole document	1
A	S.AKHTAR ET AL.: "Interactions of Antisense DNA Oligonucleotide Analogs with Phospholipid Membranes (Liposomes)." NUCLEIC ACIDS RESEARCH., vol. 19, no. 20, 1991, OXFORD GB, pages 5551-5559, XP002052617 cited in the application see the whole document	1
E	WO 96 29337 A (HYBRIDON, INC.) 26 September 1996 See the whole document, but in particular, claim 1	1-30
T	Z.ZHANG ET AL.: "Thiono Triester Modified Antisense Oligonucleotides for Inhibition of Human Cytomegalovirus in vitro." BIOORGANIC AND MEDICINAL CHEMISTRY LETTERS, vol. 6, no. 16, August 1996, pages 1911-1916, XP000677176 see the whole documents, but in particular, page 1912, compound R3	1-30
E	US 5 633 360 A (BISCHOFBERGER NORBERT ET AL) 27 May 1997 see the whole document	1-30

2

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 96/ 12530

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 24-30
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Inter ...nal Application No
PCT/US 96/12530

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